Biochemische Untersuchungen von Prenyltransferasen aus verschiedenen Ascomyceten

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Abkürzungen

µ	mikro
µm	Mikrometer
µM	Mikromolar
Abb.	Abbildung
A. fumigatus	*Aspergillus fumigatus*
A. nidulans	*Aspergillus nidulans*
A. niger	*Aspergillus niger*
A. oryzae	*Aspergillus oryzae*
A. terreus	*Aspergillus terreus*
Bp	Basenpaar
cDNA	copy DNA
Da	Dalton
DMAPP	Dimethylallyldiphosphat
DMAT	Dimethylallyltryptophan
DMATS	Dimethylallyltryptophansynthase
DMSO	Dimethylsulfoxid
DNA	Deoxyribonucleic acid (Desoxyribonukleinsäure)
DTT	1,4-Dithiothreitol
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid (Ethylendiamintetraessigsäure)
ESI	Electron spray ionisation
ETP	Epipolythiodioxopiperazin
HPLC	High performance liquid chromatography (Hochleistungsflüssigkeitschromatographie)
Hz	Hertz
kb	Kilobasen
K_M	Michaelis-Menten-Konstante
L. maculans	*Leptosphaeria maculans*
M	molar
Mb	Megabasen
MS	Massenspektrometrie
Ni-NTA	Nickel-Nitriloacetat-Agarose
NMR	Nuclear magnetic resonance (Kernresonanz-Spektroskopie)
ppm	parts per million
Tab.	Tabelle
TFA	Trifluoroacetic acid (Trifluoressigsäure)
x *g*	Erdbeschleunigung

Zusammenfassung

Aspergillus fumigatus ist ein opportunistisch pathogener Schimmelpilz und einer der Hauptauslöser invasiver Aspergillose. Zusätzlich stellt er ein wichtiges Allergen dar. Obwohl die Pathogenität dieses Pilzes von großer Bedeutung ist, ist bis jetzt noch relativ wenig über die Grundlagen dieser Pathogenität bekannt.

A. fumigatus produziert eine Vielzahl von Sekundärmetaboliten, die möglicherweise für die Pathogenität mitverantwortlich sind. U. a. bildet dieser Pilz das Epipolythiodioxopiperazin Gliotoxin, das auf Zellkulturen toxisch wirkt. Aus diesem Grund wurde spekuliert, dass Gliotoxin an der Pathogenität beteiligt sein könnte. Es kann aber nicht ausgeschlossen werden, dass noch weiterer Sekundärmetbolite einen Einfluss auf die Pathogenität von *A. fumigatus* haben. Es ist somit von großem Interesse, mehr über die genetischen Informationen und die Biosynthese von Sekundärmetaboliten, die eventuell als Virulenzfaktoren fungieren könnten, zu erfahren.

Das Gencluster der Gliotoxinbiosynthese aus *A. fumigatus* ist bekannt und besteht aus insgesamt acht Genen. Aus der Genomsequenz von *A. fumigatus* Af293 konnte ein weiteres Cluster, das drei Gene mit signifikanten Sequenzähnlichkeiten sowohl zu *gliC*, *gliP* und *gliM* aus dem Gliotoxin- als auch zu *sirC*, *sirP* und *sirM* aus dem Sirodesminbiosynthesecluster enthält, identifiziert werden. Sirodesmin ist ein weiteres Epipolythiodioxopiperazin und wird von *Leptosphaeria maculans* gebildet. Sirodesmin spielt in der Phytopathogenität dieses Pilzes eine entscheidene Rolle. Das putative Gencluster aus *A. fumigatus* enthält insgesamt acht open reading frames, das Endprodukt des Clusters ist noch unbekannt. Neben den drei schon erwähnten Genen enthält dieses Cluster ein Gen, *Afu3g12930*, das für eine putative Prenyltransferase kodiert. Die kodierende Sequenz dieses Gens wurde kloniert und in *Escherichia coli* überexprimiert. Das Protein (7-DMATS) wurde anschließend als His_6-Fusionsprotein aufgereinigt.

Untersuchungen zur Substratspezifität zeigten, dass 7-DMATS eine breite Akzeptanz besitzt und verschiedene aromatische Substrate prenyliert. L-Tryptophan wurde bei diesen Versuchen als Substrat mit der höchsten Umsetzungsrate akzeptiert. Dies war ein Indiz dafür, dass es sich bei dieser Prenyltransferase um eine Dimethylallyltryptophansynthase handelt. Durch Strukturaufklärung des

enzymatischen Produktes konnte die Position C-7 des Indolrings eindeutig als Ort der Prenylierung nachgewiesen werden.

Wie bereits erwähnt akzeptierte 7-DMATS unterschiedliche aromatische Substrate. Von den getesteten 24 Indolderivaten wurden, bis auf eine Ausnahme im Falle des 7-Methyltryptophans, alle Indolderivate von 7-DMATS akzeptiert und in ihre prenylierten Produkte umgewandelt. Strukturanalysen dieser prenylierten Indolderivate zeigte die Regiospezifität der Prenylierung. In allen Fällen katalysierte 7-DMATS die Anhängung des Prenylrestes an Position C-7 des Indolrings. Durch Optimierung der Reaktionsbedingungen konnte mit acht unterschiedlichen Indolderivaten Umsetzungsraten von 56 bis nahezu 100 % erreicht werden. Basierend auf der breiten Substratspezifität und auf den hohen Umsetzungsraten kann 7-DMATS als ein sehr gutes Werkzeug für eine chemoenzymatische Synthese von prenylierten Indolderivaten angesehen werden.

7-DMATS ist jedoch nicht nur in Hinblick auf die breite Substratspezifität, sondern auch durch eine katalytische Promiskuität ein äußerst interessantes Enzym. Diese Prenltransferase katalysiert zusätzlich zur Prenylierung von Indolderivaten auch die Hydrolyse von linearen, tryptophanhaltigen Dipeptiden und fungiert als Tryptophanaminopeptidase. Analog zu der Prenyltransferaseaktivität folgte die Peptidaseaktivität von 7-DMATS der Michaelis-Menten-Kinetik, wobei der K_M-Wert mit 350 µM für H-L-Trp-Gly-OH deutlich höher war als der K_M-Wert der Prenyltransferaseaktivität mit 137 µM für L-Tryptophan. Deutlicher als der Unterschied im K_M-Wert war jedoch der unterschiedliche Einfluss von Metallionen auf die beiden enzymatischen Aktivitäten. Es konnte gezeigt werden, dass die Prenyltransferaseaktivität von 7-DMATS metallionenunabhängig ist. Dieses Ergebnis ist vergleichbar mit den Ergebnissen aller bisher charakterisierten Indolprenyltransferasen aus Pilzen. In allen Fällen hatte die Zugabe von EDTA, einem Komplexbildner mit zweiwertigen Metallionen, nur einen geringen Einfluss auf die Prenyltransferaseaktivitäten. Im Gegensatz dazu wurde die Peptidaseaktivität von 7-DMATS durch Zugabe von EDTA deutlich gehemmt.

Untersuchungen zur Substratspezifität der Peptidaseaktivität zeigten, dass die Peptidaseaktivität flexibel bezüglich des zu hydrolysierenden Substrates ist. Für eine Hydrolyse musste Tryptophan jedoch so in dem Peptid gebunden sein, dass seine Aminogruppe frei vorliegt.

Außer 7-DMATS zeigten sieben weitere Indolprenyltransferasen eine katalytische Promiskuität. Diese Gemeinsamkeit könnte ein Indiz dafür sein, dass die Aminopeptidaseaktivität ein charakteristisches Merkmal aller Indolprenyltransferasen aus Pilzen ist. Es konnten jedoch deutliche Unterschiede in den Aminopeptidaseaktivitäten der verschiedenen Prenyltransferasen beobachtet werden. Die Peptidaseaktivitäten unterscheideten sich in Bezug auf die Hydrolyserate, auf den Einfluss von Mn^{2+} Ionen und reduzierenden Agenzien.

In dieser Arbeit wurde neben 7-DMATS die putative Prenyltransferase SirD aus der Biosynthese des Phytotoxins Sirodesmin PL aus *L. maculans* charakterisiert. Bei diesen Untersuchungen stand vor allem die Frage des natürlichen Substrates im Vordergrund. Das Gen *sirD* konnte zwar im Gencluster von Sirodesmin PL identifiziert werden, es war aber unklar, welcher Schritt in der Biosynthese durch diese Prenyltransferase katalysiert wird. Es wurde spekuliert, dass SirD entweder das erste Enzym dieses Biosyntheseweges darstellt und die Prenylierung von Tyrosin katalysiert oder dass der Biosyntheseweg mit der Bildung des zyklischen Dipeptids aus Tyrosin und Serin beginnt und SirD das Dipeptid anschließend prenyliert. Interessanterweise besitzt SirD eine hohe Sequenzähnlichkeit von 34 % zu 7-DMATS. Es wäre somit auch möglich, dass Tryptophan das natürliche Substrat von SirD ist. Für die Klärung der Frage wurde die kodierende Sequenz von *sirD* kloniert und in *E. coli* überexprimiert. Das His_6-Fusionsprotein wurde anschließend aufgereinigt und biochemisch charakterisiert. In dieser Arbeit konnte eindeutig gezeigt werden, dass SirD als Tyrosin O-Prenyltransferase fungiert und den ersten Schritt in der Biosynthese von Sirodesmin PL katalysiert. In Konsequenz zu der hohen Sequenzhomologie zu 7-DMATS prenyliert SirD zusätzlich auch Tryptophan an Position C-7 des Indolrings. Aus diesem Grund besitzt diese Prenyltransferase eine Form von Substratpromiskuität, da SirD einerseits die Bildung einer O-C Bindung, aber andererseits auch die Bildung einer C-C Bindung katalysieren kann. Diese Fähigkeit fehlt 7-DMATS und allen weiteren bis jetzt charakterisierten Indolprenyltransferasen aus Pilzen. Es konnte weiterhin gezeigt werden, dass SirD ebenfalls katalytische Promiskuität besitzt und als Aminopeptidase fungiert. Im Gegensatz zu Indolprenyltransferasen akzeptierte SirD als hydrolytisches Substrat nicht nur tryptophanhaltige Dipeptide, sondern auch ein Dipeptid aus den beiden Aminosäuren Tyrosin und Glycin.

Summary

Aspergillus fumigatus, an opportunistic pathogenic fungus, is the major reason for invasive aspergillosis and constitute also as an allergenic origin. Despite of the importance of the pathogenity of this fungus, only little is known about the background of its pathogenity.

A. fumigatus produces a series of secondary metabolites which can be alone or together responsible for the pathogenity. For example, this fungus produces gliotoxin, an epipolythiodixopiperazine, which is *in vitro* characterised as a potent agent for cell death. For this reason it was speculated, that this mycotoxin might be involved in the pathogenesis of *A. fumigatus*. It is still necessary to know more about the genetic information and biosynthesis of further secondary metabolites, which might function as virulence factors.

The biosynthetic gene cluster of gliotoxin has been identified in *A. fumigatus*. In the genome sequence of *A. fumigatus* Af293, an additional putative biosynthetic gene cluster containing three genes with significant sequence similarity to *gliC*, *gliP* und *gliM* of the gliotoxin cluster, as well as to *sirC*, *sirP* and *sirM* of the sirodesmin cluster could be identified. Sirodesmin is also an epipolythiodixopiperazine derivative, which is produced by *Leptosphaeria maculans*. Sirodesmin is implicated in the phytopathogenity of this fungus. The putative gene cluster from *A. fumigatus* contains in total eigth open reading frames. The end product of this cluster is still unknown. One gene in this cluster, *Afu3g12930* (termed hereafter *7-dmats*), which encodes for a putative prenyltransferase, could be identified. The coding region of this gene was cloned, overexpressed in *Escherichia coli* and afterwards the His_6-fusion protein was purified. The enzyme was found to catalyse the prenylation of various aromatic substances whith the highest conversation rate for L-tryptophan. This indicated that 7-DMATS functions as a dimethylallyltryptophansynthase. Structural elucidation of the enzymatic product revealed that the prenyl moiety was attached at position C-7 of the indole moiety.

As mentioned above, 7-DMATS accepted various aromatic substrates, including different tryptophan-containing cyclic dipeptides and different indole derivatives. 24 simple indole derivatives were tested and all of the substances, with an exception of 7-methyltryptophan, were accepted and converted to their prenylated products. Structural elucidation of these prenylated products proved the prenylation at position

C-7 and demonstrated that 7-DMATS catalyses regiospecifically the prenylation at position C-7 of the indole moiety. Eight different indole derivatives could be prenylated with conversion rates between 56 and 100 % under optimised conditions. Based on the broad substrate specificity and the high conversation rate, 7-DMATS can be used as an effective tool for chemoenzymatic synthesis of prenylated indole derivatives.

7-DMATS is an interesting enzyme not only due its potential as a tool for chemoenzymatic synthesis, but also its catalytic promiscuity. 7-DMATS catalyses the prenylation of indole derivatives and on the other hand the hydrolysis of linear tryptophan-containing dipeptides and functions therefore as a tryptophan aminopeptidase.

In analogy to the prenyltransferase activity, the peptidase activity of 7-DMATS followed Michaelis-Menten kinetics. A K_M value of 350 µM for H-L-Trp-Gly-OH could be determined for the peptidase activity. This is significantly higher than that of 137 µM for the prenyltransferase activity of 7-DMATS with L-tryptophan. Comparable to all other characterised indole prenyltransferases from fungi, the prenyltransferase activity of 7-DMATS is independent of the presence of metal ions. Addition of EDTA, a chelating agent for divalent metal ions, showed only low effect on the enzymatic activity. In contrast, addition of EDTA resulted in a strong decrease of the peptidase activity of 7-DMATS. Investigations on the substrate specificity of the peptidase activity showed substrate flexibility, but the importance of a tryptophan unit with a free amino group.

In addition to 7-DMATS, seven further indole prenyltransferases from fungi share also this catalytic promiscuity, which might indicate that this characteristic is shared by all indole prenyltransferases from fungi. The aminopeptidase activity of the different prenyltransferases differs from each other in the ratio of the hydrolytic activity, in the behaviours of Mn^{2+} ions and reducing agents.

In this dissertation, a putative prenyltransferase SirD from the biosynthetic gene cluster of the phytotoxin sirodesmin of *L. maculans* was also characterised. Focus of these experiments was the question of the natural substrate of SirD. Until now, it was not clear whether SirD catalyses the first step and prenylates tyrosine or catalyses the prenylation of the cyclic dipeptid of serine and tyrosine and therefore represents the second step in the biosynthesis of sirodesmin. Based on a significant sequence

homology to 7-DMATS it was also possible that SirD prenylates tryptophan. To answer this question, the coding region of *sirD* was cloned and overexpressed in *E. coli* and the His_6-fusion protein was purified and biochemical characterised. It could be shown that SirD functions as a tyrosine O-prenyltransferase and catalyses the first pathway-specific step in the biosynthesis of sirodesmin. In consequence of the high sequence similarity to 7-DMATS, SirD prenylates besides tyrosine also tryptophan at position C-7 of the indole moiety. This finding demonstrated clearly the substrate promiscuity of SirD. SirD showed also catalytic promiscuity and functions as an aminopeptidase. Differing from indole prenyltransferases, SirD accepted not only tryptophan-containing dipeptides, but also a dipeptide of tyrosine and glycine as substrate for the hydrolytic reaction.

1 Einleitung

1.1 Ascomyceten

Ascomyceten bilden zusammen mit den Basidiomyceten das Unterreich der Dikarya. Etwa 98 % der beschriebenen Pilzspezies gehören diesem Unterreich an (James *et al.,* 2006).

Charakteristisch für Ascomyceten ist die Ausbildung eines schlauchförmigen Fruchtkörpers, dem sog. Ascus. Aufgrund der Form dieses Fruchtkörpers werden sie auch als Schlauchpilze bezeichnet.

Ascomyceten besitzen eine sehr große Bedeutung für den Menschen. Einerseits werden sie traditionell in der Lebensmittelherstellung eingesetzt und sind in der Medizin beispielsweise als Arzneistoffproduzenten sehr wichtig, andererseits besitzen sie aber auch ein sehr hohes pathogenes Potential. Unter den Ascomyceten befinden sich neben mensch- und tier- auch einige pflanzenpathogene Arten.

1.1.1 Die Gattung *Aspergillus*

1729 veröffentlichte der italienische Botaniker Pier Antonio Micheli sein Werk „ Nova plantarum genera“. In diesem Werk beschrieb er insgesamt etwa 1900 Arten, von denen 1400 zum ersten Mal erwähnt wurden (Micheli, 1729). Die meisten dieser neu beschriebenen Arten gehörten zu den niederen Pflanzen, darunter befanden sich auch etwa 900 Pilzarten. Unter diesen Pilzen entdeckte er eine Gattung, deren mikroskopisches Bild ihn an einen Weihwasserwedel, einen Aspergill, erinnerte. Aus diesem Grund nannte er diese Gattung *Aspergillus* (Micheli, 1729). Durch die außergewöhnliche Form des konidien-abschnürenden Mycelköpfchens, das an eine Gießkanne erinnert, bekam dieser Pilz den deutschen Namen Gießkannenschimmelpilz, der heutzutage noch gelegentlich verwendet wird.

Die Gattung *Aspergillus* gehört zur Familie der Trichocomaceae (Pitt *et al.,* 2000) und ist weltweit verbreitet. Sie kommt in Böden und Komposthäufen vor und ist unabhängig von tierischen oder menschlichen Wirten. Diese Gattung beinhaltet mehr als 180 Arten und ist ökonomisch sehr wertvoll (Ward *et al.,* 2006). Viele Arten

werden in der Industrie zur Produktion von homologen und heterologen Enzymen verwendet (Fawole & Odunfa, 2003; Wang *et al.,* 2005). Ein Beispiel für die industrielle Nutzung von homologen Enzymen sind Pektinasen, die vor allem in der Lebensmittelindustrie zur Zubereitung von Säften und Weinen eingesetzt werden (Semenova *et al.,* 2006). Beispiele für Enzyme, die in verschiedenen *Aspergillus*-Arten heterolog exprimiert und industriell genutzt werden, sind Lysozym aus Hühnereiweiß, humanes Interleukin-6 und humanes Lactoferrin (Wang *et al.,* 2005). Zusätzlich spielen die verschiedenen *Aspergillus*-Arten in der Umwelt aufgrund des Abbaus von pflanzlichen Polysacchariden eine große Rolle (de Vries *et al.,* 2000; de Vries, 2003). Auch wenn verschiedene *Aspergillus*-Arten eine Bedeutung für die Produktion von Lebensmitteln und von verschiedenen Proteinen besitzen, sind andere Arten wie *Aspergillus fumigatus*, *Aspergillus flavus*, *Aspergillus terreus* und *Aspergillus niger* als Krankheitserreger bekannt (Anderson *et al.,* 2003). Charakteristisch für die verschiedenen pathogenen *Aspergillus*-Arten ist u. a. eine relativ hohe Hitzetoleranz (Tekaia & Latgé, 2005). Für ein besseres Verständnis dieser Pathogenitätsmechanismen, aber auch um einen Einblick in die zellulären Mechanismen zu bekommen, wurde vor einigen Jahren mit Untersuchungen von einzelnen Genen bzw. kompletten Genomen einiger Arten dieser Gattung begonnen und teilweise schon beendet (Fedorova *et al.,* 2008; Galagan *et al.,* 2005; Machida *et al.,* 2005; Nierman *et al.,* 2005; Pel *et al.,* 2007)

Im Folgenden werden die bedeutsamen vier *Aspergillus*-Arten *A. niger*, *A. oryzae*, *A. nidulans* und *A. fumigatus* vorgestellt, wobei der Schwerpunkt auf *A. fumigatus* gelegt wird.

1.1.1.1 *Aspergillus niger*

A. niger besitzt große biotechnologische Bedeutung und wird für die Produktion von verschiedenen Lebensmittelzusätzen, aber auch zur Produktion von heterologen Enzymen eingesetzt (Punt *et al.,* 2002). Als wichtigster Lebensmittelzusatz ist hier Zitronensäure, die das erste Produkt des Zitronensäurezyklus darstellt, zu nennen. Mit Hilfe von geeigneten Produktionsstämmen läßt sich mittlerweile eine fast quantitative Umsetzung der Kohlenstoffquelle in Zitronensäure erreichen (Karaffa & Kubicek 2003). Des Weiteren ist *A. niger* in der Lage mit Schwermetallen kontaminierte Böden zu sanieren (Ren *et al.,* 2009).

1.1.1.2 *Aspergillus oryzae*

A. oryzae wird bereits seit etwa 1000 Jahren in der traditionellen japanischen Küche für verschiedene Fermentationsprozesse von Nahrungsmitteln wie Sake oder Sojasauce eingesetzt. Heutzutage findet *A. oryzae* aber auch Anwendung in der Produktion von rekombinanten Enzymen (Abe *et al.,* 2006). Das Genom dieses Schimmelpilzes wurde bereits komplett entschlüsselt. Es besitzt eine Größe von 37 Mb und enthält 12074 Gene (Machida *et al.,* 2005).

1.1.1.3 *Aspergillus nidulans*

A. nidulans ist, genau wie *A. oryzae,* ein nicht pathogener Schimmelpilz. Dieser Pilz wurde ursprünglich dazu genutzt, die Funktionen von Tubulin und Mikrotuboli auf molekularer Ebene zu charakterisieren (Morris *et al.,* 1979). Aufgrund seiner nahen Verwandtschaft zu *Aspergillus*-Arten mit medizinischer und industrieller Signifikanz findet er heute häufig Verwendung in der Molekularbiologie. Das Ziel ist es, gewonnene Erkenntnisse auf andere Arten übertragen zu können. Das Genom von *A. nidulans* hat eine Größe von etwa 30 Mb. In dem Genom sind bereits mehr als 9500 Gene identifiziert worden (Galagan *et al.,* 2005).

1.1.1.4 *Aspergillus fumigatus*

A. fumigatus gehört zu den weitverbreitesten Spezien auf der Erde und kommt vor allem in Böden oder Komposthäufen vor. Dieser Schimmelpilz verdankt seinen Namen „fumigatus“ (fumus, lat. der Rauch) seinen rauchgrünen Sporen. Er ernährt sich saprophytisch, d.h. von sich zersetzendem organischem Material und hat somit seine primäre ökologische Bedeutung im Recycling von Stickstoff und Kohlenstoff (Wilson *et al.,* 2002). Für diese Lebensweise besitzt *A. fumigatus* verschiedene Enzyme, die die Polysaccharide der Pflanzenzellwand verdauen können (de Vries & Visser, 2001). Er besitzt jedoch keine Enzyme für einen Ligninabbau, so dass dieser Pilz beim Holzabbau keine Rolle spielt (Tekaia & Latgé, 2005).

Im Gegensatz zu anderen *Aspergillus*-Arten weist *A. fumigatus* eine hohe Thermotoleranz auf. Er kann bei Temperaturen bis 55 °C wachsen und überlebt Temperaturen bis 70 °C (Beffa *et al.,* 1998; Ryckeboer *et al.,* 2003). Aus diesem Grund ist er auch ein wesentlicher Bestandteil der Mikroflora von Komposthäufen. Für diese Spezies war lange Zeit nur eine vegetative Vermehrung mit Hilfe von

haploiden, asexuellen Sporen bekannt (Ward *et al.,* 2006), mittlerweile konnte aber auch eine sexuelle Vermehrungsform beobachtet werden (O'Gorman *et al.,* 2009).

A. fumigatus ist sowohl für Menschen als auch für Tiere ein opportunistisches Pathogen. Er ist in der Lage sehr stark zu sporulieren und bildet pro Konidienträger tausende Konidien. Diese Konidien sind mit einem Durchmesser von 2 bis 3 µm sehr klein, so dass sie bis in die Lungen-Alveolen vordringen können. Durch die Vielzahl der gebildeten Konidiosporen ist die Konzentration an Sporen in der Luft sehr hoch, so dass der Mensch über die Lunge täglich mehrere hundert *A. fumigatus* Konidien aufnimmt (Latgé, 1999). Für Menschen mit einem intakten Immunsystem hat dies in der Regel keinen Einfluss, wohingegen bei immungeschwächten Patienten ein Eindringen von Sporen in die Lunge zu gesundheitlichen Problemen führen kann.

In den letzten Jahren hat sich, bedingt durch ein vermehrtes Auftreten von immungeschwächten Patienten, die Anzahl von invasiven Pilzinfektionen deutlich erhöht (Cramer, Jr. *et al.,* 2006). Vor allem invasive Pilzinfektionen ausgelöst durch *A. fumigatus*, sog. invasive Aspergillose, sind verantwortlich für die Erkrankung und im schlimmsten Fall für den Tod dieser Patienten. Todesraten von über 50 % bei Hochrisikogruppen wie Patienten die an Leukämie erkrankt sind, wurden beobachtet (Hohl & Feldmesser, 2007). Auch andere Aspergillen wie *A. terreus* oder *A. niger* können als Pathogen fungieren, *A. fumigatus* ist hierbei jedoch der bedeutestenste Verursacher. *A. fumigatus* löst nicht nur invasive Aspergillose aus sondern stellt auch ein wichtiges Allergen dar (Casadevall & Pirofski, 1999; Denning, 1998; Greenberger, 2002). Die Interaktion von *A. fumigatus* aus der Luft mit dem Immunsystem wird verstärkt mit dem Auftreten schweren Asthmas und Sinnusitis in Verbindung gebracht (Zureik *et al.,* 2002).

Aufgrund der großen medizinischen Bedeutung von *A. fumigatus* ist es sehr wichtig, mehr über die Pathologie dieses Pilzes zu erfahren. Trotz der kompletten Entschlüsselung des Genoms (Nierman *et al.,* 2005) sind die Ursachen der ausgeprägten Pathogenität von *A. fumigatus* noch weitesgehend unbekannt. Verschiedene Besonderheiten von *A. fumigatus* wurden in diesem Zusammenhang diskutiert. So ist er im Vergleich zu anderen pathogenen *Aspergillus*-Arten thermotoleranter, was ihm nicht nur einen Vorteil bei der Ausfüllung von ökologischen Nischen bringt, sondern auch das Wachstum in Respirationsorganen von Säugetieren begünstigt. Auch Größe und Morphologie der Konidien wurde in Bezug

auf die Pathogenität diskutiert, bis jetzt wurden aber noch keine Beweise für diese Hypothesen erbracht (Hohl & Feldmesser, 2007).

Das Genom des klinischen Isolates Af293 wurde bereits komplett entschlüsselt und besitzt eine Größe von 29,4 Mb. Es besteht aus acht Chromosomen mit Größen zwischen 1,8 und 4,9 Mb, die 9926 putative Gene enthalten. Die Gene sind im Durchschnitt 1431 Bp groß und enthalten durchschnittlich 1,8 Introns je Gen, der G+C-Gehalt liegt bei 49,9 % (Nierman *et al.,* 2005).

1.1.2 Die Gattung *Leptosphaeria*

Die Gattung *Leptosphaeria* gehört zur Familie der Leptosphaeriaceae (Schoch *et al.,* 2006) und ist sehr artenreich. Sie umfasst mehr als 1600 Taxa wobei viele phytopathogen sind und auf unterschiedlichsten Pflanzen leben (Mendes-Pereira *et al.,* 2003). Viele dieser Arten sind dabei nur auf eine einzige Pflanzengattung oder sogar nur auf eine Pflanzenart spezialisiert. Sie besiedeln Stängel und Äste sowie Blätter und Früchte.

1.1.2.1 *Leptosphaeria maculans*

L. maculans ist die Hauptfruchtform des Erregers der Wurzelhals- und Stängelfäule bei Raps (*Brassica napus*) und das mit Abstand weltwirtschaftlich wichtigste Rapspathogen (Howlett *et al.,* 2001). Die Bezeichnung *Phoma lingom* bezieht sich auf die ungeschlechtliche Form des Pilzes. Der Pilz wurde erstmalig 1791 von TODE auf vertrockneten Rotkohlstängeln entdeckt. Er wurde aufgrund des Wachstums auf totem Pflanzenmaterial fälschlicherweise als Saprophyt eingeordnet und als *Sphaeria lingam* bezeichnet (Tode, 1791). Sehr viel später isolierte DESMAZIERE den gleichen Pilz von lebendem Pflanzenmaterial und klassifizierte ihn in den Genus *Phoma* ein (Desmaziere, 1849). Die Taxonomie und Bezeichnung von *L. maculans* ist verwirrend, da dieser Pilz verschiedene Spezien umfasst, die sich morphologisch sehr ähnlich sind (Elliott *et al.,* 2007). Die verschiedenen Stämme werden anhand ihrer Fähigkeit die typischen Symptome im Stängel- und Wurzelhalsbereich auszulösen in zwei Gruppen unterteilt. Für diese beiden Gruppen gibt es keine einheitliche Nomenklatur. Stämme der ersten Gruppe werden nur als Gruppe A, aggressive oder hochvirulente *L. maculans* Stämme bezeichnet, wohingegen

Stämme der zweiten Gruppe als Stämme der Gruppe B, nichtaggressive oder schwach virulente *L. maculans* Stämme bezeichnet werden (Howlett *et al.,* 2001).

Die Fähigkeit von *L. maculans* sich sowohl asexuell über Pyknidiosporen als auch sexuell über Ascosporen zu vermehren, stellt ein großes Verbreitungspotential dar. Nach der Ernte bildet der Pilz auf abgestorbenem Pflanzenmaterial schwarze, rundliche Fruchtkörper mit einem Durchmesser von 300-500 µm, sog. Pseudothezien, mit hyalinen septierten Pseudoparaphysen. Die zylindrischen bis elipsoiden, gelb-braunen, haploiden Ascosporen werden bei Niederschlag freigesetzt und können mit Hilfe des Windes über große Strecken transportiert werden (Williams, 1992). Die Infektion des Keimlings erfolgt durch Eindringen der Ascosporen über die Stomata in die Kotyledonen oder Primärblätter. Diese Ascosporen rufen dort graue bis schmutzig-weiße Läsionen hervor. In dem Keimling wird der Pilz nekrophytisch und bildet in dem abgestorbenen Gewebe asexuelle Fruchtkörper, sog. Pyknidien (Hammond & Lewis, 1987). Pyknidiosporen besiedeln mit Hilfe von Niederschlag weitere Blattetagen oder auch den Wurzelhals benachbarter Pflanzen. Der Befall mit Ascosporen wird als Primärinfektion bezeichnet, wohingegen die Verbreitung mit Hilfe von Pyknidiosporen als Sekundärinfektion bezeichnet wird (Williams, 1992).

Die Größe des Genoms von *L. maculans* ist mit etwa 34 Mb vergleichbar zu anderen Ascomyceten. Das Genom liegt verteilt auf 16 Chromosomen mit Größen zwischen 0,7 und 3,5 Mb. (Elliott *et al.,* 2007). Unter diesen 16 Chromosomen sind ein paar kleinere Chromosome mit Größen zwischen 0,65 bis 0,95 Mb, die nicht auf die nächste Generation nach Mendel weitergegeben werden. Diese Chromosomen enthalten repetetive Bereiche und haben keinen Einfluss auf saprophytische oder parasitische Fähigkeiten (Leclair *et al.,* 1996).

1.2 Sekundärmetabolite

Sekundärmetabolite sind niedermolekulare Substanzen, die von Pflanzen, Bakterien und Pilzen gebildet werden. Sie sind scheinbar für Wachstum und Reproduktion des Organismus nicht unbedingt notwendig. Die Zahl der identifizierten Sekundärmetabolite ist sehr hoch, alleine aus Pflanzen sind bereits mehr als 200.000 Sekundärmetabolite bekannt (Dingermann *et al.,* 2004b).

Pilzliche Sekundärmetabolite wie Penicilline oder Cephalosporine weisen häufig antimikrobielle Aktivitäten auf (Elander, 2003). Es wird vermutet, dass die Produktion dieser Antibiotika dem Produzenten einen Vorteil in der Besiedelung von ökologischen Nischen bringt (Vining, 1990).

Doch Sekundärmetabolite sind nicht nur, wie im Fall der verschiedenen Antibiotika, nützlich für medizinische Anwendungen, sondern können auch toxisch und karzinogen für Menschen und Tiere sein. Beispiele hierfür sind verschiedene Mykotoxine. Mykotoxine bezeichnen eine Gruppe von bioaktiven Sekundärmetaboliten aus Pilzen, die chemisch sehr unterschiedlich sein können und eine relativ kleine Größe von weniger als 700 Da besitzen (Abarca *et al.,* 2000). Die meisten Mykotoxine werden von Pilzen der Gattungen *Aspergilllus*, *Penicillium* und *Fusarium* gebildet, wobei Aflatoxine, die von *A. flavus*, *A. parasiticus* und *A. nomius* produziert werden, die größte Bedeutung besitzen. Aflatoxine sind lebertoxisch, karzinogen, teratogen und mutagen (Ward *et al.,* 2006).

A. fumigatus produziert viele Sekundärmetabolite wie Ergotalkaloide vom Clavin-Typ. Beispiele hierfür sind Elymoclavin, Festuclavin und Fumigaclavin (Flieger *et al.,* 1997; Spilsbury & Wilkinson, 1961), aber auch Fumagillin, Gliotoxin, Fumitremorgine oder Verruculogen, die möglicherweise für seine Pathogenität mitverantwortlich sein können (Latgé, 1999).

Charakteristisch für Gene, die in die Synthese von Sekundärmetaboliten von Pilzen involviert sind, ist die Bildung einer zusammenhängenden Gruppe. Diese Gruppe wird als Cluster bezeichnet (Keller & Hohn, 1997). Diese Cluster sind nur teilweise konserviert. Das Genom von *A. fumigatus* enthält auf den acht Chromosomen mindestens 26 von solchen Clustern mit Polyketidsynthase-, nichtribosomalen Peptidsynthetase- und/oder Prenyltransferase-Genen. Von diesen 26 Clustern konnten nur 13 orthologe Cluster in *A. oryzae* oder *A. nidulans* identifiziert werden (Nierman *et al.,* 2005).

1.2.1 Epipolythiodioxopiperazine

Verschiedene Klassen von pilzlichen Toxinen sind bereits gut untersucht worden. Zu diesen Klassen gehören Polyketide, zyklische Peptide, Alkaloide und Sesquiterpenoide (Keller *et al.,* 2005). Daneben ist eine weitere Klasse von Toxinen,

die sog. Epipolythiodioxopiperazine, bekannt. Diese Toxine sind charakterisiert durch eine interne Disulfidbrücke (Gardiner *et al.,* 2005). Das Grundgerüst, der Diketopiperazinring, leitet sich von zyklischen Dipeptiden ab (Mullbacher *et al.,* 1986). Mindestens 14 verschiedene Epipolythiodioxopiperazine sind bekannt, wobei die verschiedenen Substanzen dieser Klasse einerseits durch die unterschiedlichen Aminosäuren, aber andererseits auch durch Modifikationen dieser Aminosäuren zustande kommen. Alle aus Pilzen isolierte Epipolythiodioxopiperazine enthalten mindestens eine aromatische Aminosäure (Gardiner *et al.,* 2005). Bis jetzt sind zwei mögliche Arten der Toxizität von Epipolythiodioxopiperazinen bekannt. Zum Einen agieren sie als redoxaktive Toxine, indem sie reaktive Sauerstoffverbindungen, die zwischen ihrer oxidierten und ihrer reduzierten Form wechseln, produzieren (Munday, 1987), zum Anderen bilden sie gemischte Disulfide mit freien Thiolgruppen von Proteinen (Chai & Waring, 2000). Verschiedene Pilze produzieren Epipolythiodioxopiperazine, darunter Stämme aus der Klasse der Deuthideomycetes, Euromycetes aber auch aus der Klasse der Saccharomycetes. Dabei ist die Produktion von Epipolythiodioxopiperazinen unter den verschiedenen pilzlichen Spezies einer Gattung nicht gleichmäßig verteilt. So produziert *A. fumigatus* Gliotoxin, wohingegen für andere *Aspergillus*-Stämme eine Gliotoxinsynthese nicht nachgewiesen werden konnte. Das gleiche Phänomen ist bei der Gattung *Leptosphaeria* zu beobachten. *L. maculans* produziert Sirodesmin PL, aber *Leptosphaeria biglobosa* nicht (Gardiner *et al.,* 2005). Es können aber auch weiter entfernte Pilze das gleiche Epipolythiodioxopiperazin synthetisieren. So wird Gliotoxin von verschiedenen nicht miteinander verwandten Pilzen wie *A. fumigatus*, *Penicillium* ssp., *Candida* spp. oder *Trichoderma* spp. produziert (MacDonald & Slater, 1975; Shah & Larsen, 1991).

Wichtigste Vertreter dieser Toxinklasse sind Gliotoxin und Sirodesmin PL, auf die im Folgenden näher eingegangen wird.

1.2.1.1 Gliotoxin

Gliotoxin wurde in verschiedenen Pilzstämmen, u.a. in dem opportunistisch pathogenen Stamm *A. fumigatus,* identifiziert (Glister & Williams, 1944). Es wurde spekuliert, dass dieses Mykotoxin in die Pathogenität von *A. fumigatus* involviert ist (Kwon-Chung & Sugui, 2009). In tierischen Zelllinien konnte gezeigt werden, dass

Gliotoxin sowohl apoptotischen als auch nekrotischen Zelltod bewirkt (Hurne *et al.,* 2002; Waring *et al.,* 1988). Aus diesem Grund besaß eine Identifizierung der in die Biosynthese dieses Mykotoxins involvierten Gene eine große Bedeutung.

2005 wurde zum ersten Mal das putative Gliotoxingencluster aus *A. fumigatus* veröffentlicht (dargestellt in Abb. 1.1). Dieses Cluster umfasst 28 kb und enthält 12 Gene, darunter ein Gen (*gliJ),* das für eine Dipeptidase, ein Gen (*gliP),* das für eine nicht-ribosomale Peptidsynthetase kodiert und zwei Gene (*gliN* und *gliM*) die für zwei Methyltransferasen kodieren. Des Weiteren konnte ein Gen (*gliA*), das für einen Transporter und ein Gen (*gliZ*), das für einen Transkriptionsregulator kodiert, identifiziert werden. Ebenfalls konnte ein Gen (*gliT*), dessen Genprodukt eine Thioredoxinreduktase ist, identifiziert werden. Diese Thioredoxinreduktase ist notwendig für die Bildung der Disulfidbindung. Zu dem Gliotoxincluster gehören zusätzlich noch zwei Gene (*gliC* und *gliF*) die für Cytochrom-P450 Enzyme kodieren und ein Gen (*gliK*) zu dem keine homologen Gene gefunden werden konnten (Gardiner & Howlett, 2005).

Abb. 1.1: Putatives Gliotoxinbiosynthesecluster aus *A. fumigatus* (Gardiner & Howlett 2005)

Der putative Gliotoxinbiosyntheseweg aus *A. fumigatus* ist in Abb. 1.2 dargestellt. Die Biosynthese beginnt mit der Kondensation von Serin und Phenylalanin, katalysiert durch die nicht-ribosomale Peptidsyntethase GliP, anschließend erfolgen eine Serie von Oxidationen, Sulfurisationen und Methylierungen. In diesem Biosyntheseweg konnte bis jetzt nur das erste Intermediat, ein zyklisches Dipeptid aus Serin und Phenylalanin, identifiziert werden (Bose *et al.,* 1968; Gardiner & Howlett, 2005).

Fünf verschiedene Arbeitsgruppen führten unterschiedliche Arbeiten zu diesem Cluster durch. Sie schalteten entweder *gliP* (Cramer, Jr. *et al.,* 2006; Kupfahl *et al.,* 2006; Spikes *et al.,* 2008; Sugui *et al.,* 2007) oder *gliZ* aus (Bok *et al.,* 2006) und beobachteten den Effekt auf die Gliotoxinbiosynthese. Durch diese Arbeiten konnte

gezeigt werden, dass dieses Cluster wirklich für die Biosynthese von Gliotoxin verantwortlich ist. Die mutierten Stämme waren in keinem Fall in der Lage Gliotoxin zu synthetisieren. Erstaunlicherweise waren die Ergebnisse von Tierversuchen bezüglich der Toxizität von Gliotoxin nicht einheitlich. Drei dieser Arbeitsgruppen konnten keinen Einfluss von Gliotoxin auf die Virulenz von *A. fumigatus* beobachten, wohingegen die anderen beiden Arbeitsgruppen das Gegenteil postulierten. Die gegensätzlichen Ergebnisse basierten auf der unterschiedlichen Verwendung von immunosupprimierten Mäusen. Bei Verwendung von Mäusen, die mit Hilfe von Cyclophosphamiden und Corticosteroiden immunosupprimiert wurden konnte kein toxischer Effekt von Gliotoxin beobachtet werden. Im Gegensatz dazu konnte bei Mäusen, die nur mit Corticosteroiden immunosupprimiert wurden, eindeutig gezeigt werden, dass Gliotoxin ein wichtiger Virulenzfaktor von *A. fumigatus* ist (Kwon-Chung & Sugui, 2009).

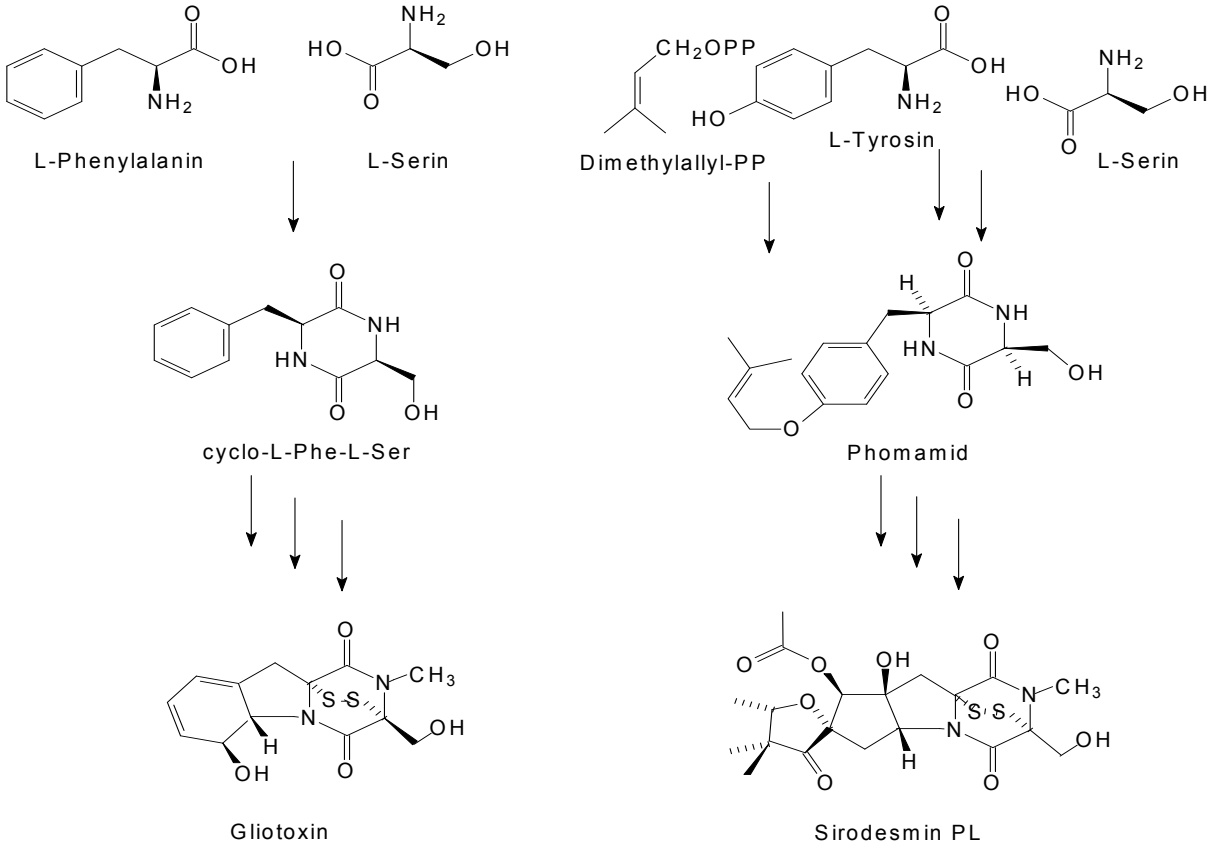

Abb. 1.2: Vereinfachte Biosynthesewege von Gliotoxin und Sirodesmin PL mit den identifizierten Intermediaten cyclo-L-Phe-L-Ser für Gliotoxin und Phomamid für Sirodesmin PL

1.2.1.2 Sirodesmin PL

Sirodesmin PL wird in großer Menge von *L. maculans* ausgeschleust und umfasst 50-70 % des phytotoxischen Extraktes dieses Pilzes (Howlett *et al.,* 2001). Sirodesmin PL ist nicht nur ein Pflanzenpathogen, sondern zeigt auch antibakterielle und antivirale Aktivitäten (Rouxel *et al.,* 1988). Die Rolle dieses Epipolythiodioxopiperazins während des Befalls von Raps ist noch unklar. Der Nachweis des Phytotoxins ist abhängig von den Wachstumsbedingungen der Pflanze (Pedras & Séguin-Swartz, 1992; Sock & Hope, 1999).

Das Sirodesminbiosynthesegencluster wurde 2004 von GARDINER ET AL. identifiziert. Das Cluster enthält 18 Gene und hat eine Größe von 59 kb (dargestellt in Abb. 1.3). Einigen Genen dieses Clusters konnten durch Homologievergleiche mit anderen schon charakterisierten Genen mögliche Funktionen in der Biosynthese von Sirodesmin PL zugeordnet werden. Zusätzlich zu einem Gen (*sirD*), das für eine Prenyltransferase und einem Gen (*sirT*), das für eine Thioredoxinreduktase kodiert, enthält dieses Cluster ein Gen (sirP), das für eine nicht-ribosomale Peptidsynthetase kodiert. Inaktivierung von *sirP* führte zu einer Blockierung der Sirodesminbiosynthese (Gardiner *et al.,* 2004). Auch ein Gen (*sirH*), das für eine Acetyltransferase kodiert, konnte in diesem Cluster identifiziert werden. Diese Acetyltransferase katalysiert den letzten Schritt in der Biosynthese. Es wurde auch vermutet, dass eine Methyltransferase in die Biosynthese von Sirodesmin PL involviert sein muss. Dementsprechend enthält das Cluster zwei Gene (*sirM* und *sirN*), die möglicherweise für eine O-Methyltransferase und eine weitere, nicht näher zugeordnete Methyltransferase, kodieren (Gardiner *et al.,* 2004). Für den Export von Toxinen und für den Selbstschutz der Pilze sind Transporterproteine wichtig, so dass deren Gene in den Clustern von Sekundärmetaboliten von Pilzen zu finden sind (Del *et al.,* 2000). Auch in dem Sirodesmincluster konnte ein Gen (*sirA*), das für einen ABC-Transporter kodiert, identifiziert werden. Neben diesen Genen finden sich in diesem Cluster weitere Gene, deren Genprodukte vermutlich Oxidations- und Reduktionsreaktionen katalysieren und die charakteristisch sind für Cluster von pilzlichen Sekundärmetaboliten. Zusätzlich enthält dieses Cluster aber auch Gene, bei denen eine Zuordnung der Funktion noch nicht möglich ist, da deren Homologen noch nicht näher charakterisiert sind (Gardiner *et al.,* 2004).

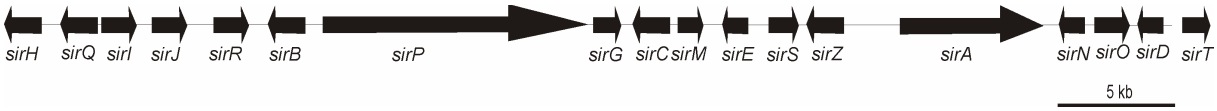

Abb. 1.3: Putatives Sirodesminbiosynthesecluster aus *L. maculans* (Gardiner *et al.,* 2004)

Der Biosyntheseweg von Sirodesmin ist in Abb. 1.2 dargestellt und beginnt mit der Kondensation von Tyrosin und Serin katalysiert durch die nicht-ribosomale Peptidsynthetase SirP. Tyrosin wird vorher wahrscheinlich durch die Prenyltransferase SirD prenyliert. Phomamid als erstes Intermediat dieses Biosyntheseweges konnte eindeutig identifiziert werden (Ferezou *et al.,* 1980). Aufgrund der Ähnlichkeit der Biosynthesewege von Sirodesmin PL und Gliotoxin sind in Abb. 1.2 beide Wege nebeneinander dargestellt.

1.2.2 Alkaloide

Alkaloide sind stickstoffhaltige Substanzen aus dem Sekundärstoffwechsel von Pflanzen, Tieren oder Pilzen, die meist alkalisch reagieren und bei denen häufig der Stickstoff ein Teil eines Ringsystems darstellt. Die Unterteilung der Alkaloide in verschiedene Gruppen kann nach unterschiedlichen Kriterien durchgeführt werden, am gebräuchlisten ist jedoch eine Einteilung nach Art des Heterozyklus.

1.2.2.1 Alkaloide aus Pflanzen

Alkaloide, die als Arzneistoffe Verwendung finden, werden vor allem von Vertretern der Familie der Apocynaceen, der Loganiaceen und der Rubiaceen gebildet. Ein Beispiel für Alkaloide, die pharmazeutisch genutzt werden, ist Reserpin aus *Rauvolfia serpentina*. Reserpin besitzt vor allem blutdrucksenkende und sedierende Wirkung. Weitere pharmazeutisch bedeutsame Alkaloide sind Chinin und Chinidin aus *Cinchona pubescens*. Chinin wird vor allem als Malariamittel eingesetzt und Chinidin findet Anwendung als Antiarrhythmikum. Zwei bedeutende Zytostatika aus der Klasse der Indolalkaloide sind Vinblastin und Vincristin. Beide kommen im Madagaskar-Immergrün (*Catharantus roseus*) vor. Vinblastin und Vincristin reagieren mit den Mikrotuboli und hemmen dabei die Bildung des Spindelapparates. Weitere

Beispiele für Alkaloide aus Pflanzen sind Yohimbin aus *Aspidosperma quebracho-blanco* und Vincamin aus verschiedenen *Vinca*-Arten. Yohimbin wird u. a. bei Asthma, Atemstörungen und Bronchitis eingesetzt. Vincamin hingegen verbessert die Hirndurchblutung und vermindert Ödeme. Aus diesem Grund wird Vincamin bei zerebraler Mangeldurchblutung und bei bestimmten unfallbedingten Ausfallerscheinungen des Gehirns eingesetzt (Dingermann *et al.,* 2004a). Die Strukturen der in diesem Abschnitt vorgestellten Alkaloide sind in Abb. 1.4 dargestellt.

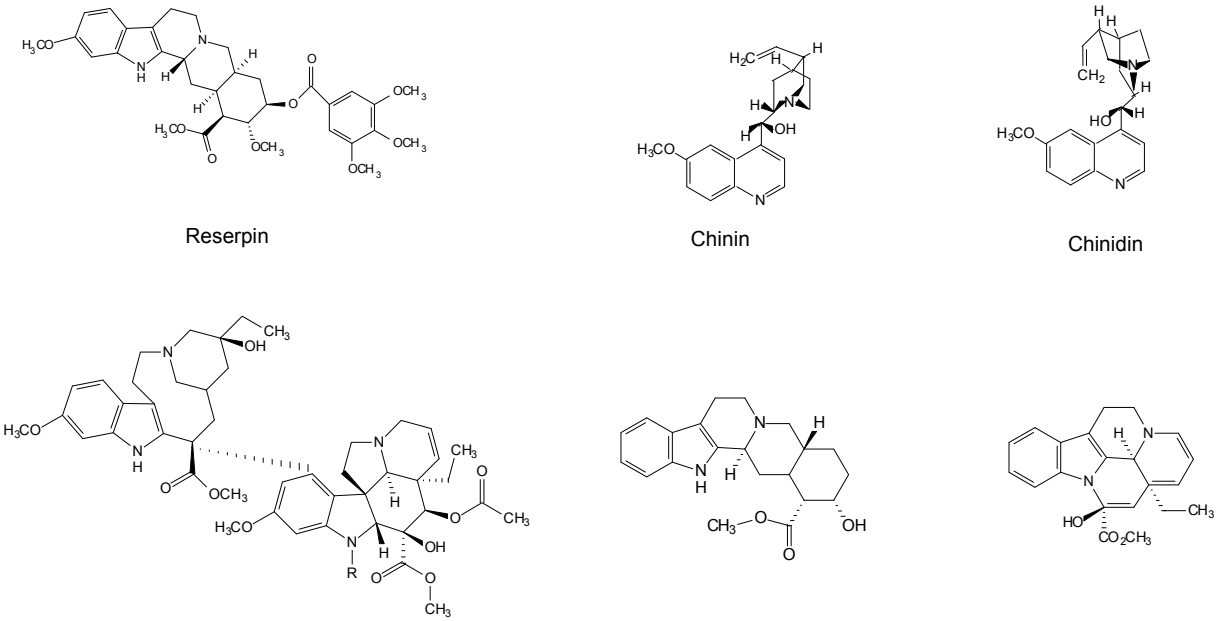

Abb. 1.4: Strukturen einiger Alkaloide aus Pflanzen

1.2.2.2 Prenylierte Indolalkaloide aus Pilzen

Charakteristisch für Indolalkaloide ist ein Indol- oder Indolingrundgerüst, das sich meistens von der Aminosäure Tryptophan ableitet. Prenylierte Indolalkaloide sind Hybrid-Naturstoffe aus einem aromatischen und einem Terpenbestandteil. Synthetisiert werden diese Indolalkaloide vor allem durch Ascomyeten der Gattungen *Claviceps*, *Penicillium* und *Aspergillus* (Stocking *et al.,* 2000; Williams *et al.,* 2000). Sie besitzen oft biologische Aktivitäten, die sie von ihren nicht prenylierten Vorstufen unterscheiden (Usui *et al.,* 1998). Prenylierte Indolalkaloide aus Pilzen besitzen sehr viele unterschiedliche chemische Strukturen. Sie zeigen unterschiedlichste pharmakologische Aktivitäten, können aber auch toxisch wirken. In Abb. 1.5 sind die

Strukturen von verschiedenen, prenylierten Indolalkaloiden aus unterschiedlichen Pilzen dargestellt.

Ergotamin und verwandte Ergopeptine, die von *Claviceps purpurea* produziert werden, führten im Mittelalter durch Kontamination von Getreide zu dem sog. Antoniusfeuer (Bennett & Klich, 2003; Flieger *et al.,* 1997; Floss 1976; Hoffmeister & Keller, 2007). Heutzutage wird Ergotamin und auch sein Derivat Dihydroergotamin in der Migränetherapie eingesetzt (Goadsby, 2003; Saper & Silberstein, 2006).

Fumitremorgin B ist ein Mykotoxin und wird von *A. fumigatus* gebildet. Die Tryprostatine A und B und auch Fumitremorgin C sind Intermediate aus der Biosynthese von Fumitremorgin B und zeigen pharmazeutisch interessante Aktivitäten. Die Diketopiperazine Tryprostatine A und B (Cui *et al.,* 1996) weisen Zytotoxizität gegenüber verschiedenen Krebszelllinien auf (Cui *et al.,* 1995; Kondoh *et al.,* 1998; Zhao *et al.,* 2002) und sowohl Tryprostatin A als auch Fumitremorgin C hemmen das BCRP (breast cancer resistance protein), einen ABC-Transporter, der Brustkrebszellen eine Multidrugresistenz gegenüber Zytostatika verleiht. Dadurch kann die Resistenz von einigen Tumorzelllinien gegenüber Zytostatika aufgehoben werden (Jain *et al.,* 2008; Rabindran *et al.,* 2000; Zhou *et al.,* 2008). Fumigaclavin C aus *Aspergillus*- und *Penicillium*-Stämmen (Cole *et al.,* 1977; Janardhanan *et al.,* 1984; Vinokurova *et al.,* 2003) hemmt die TNF-α Produktion und die Anheftung von Lymphozyten an extrazelluläre Matrizen (Zhao *et al.,* 2004), während die Terrequinon-Alkaloide aus verschiedenen *Aspergillus*-Stämmen antitumorale, antidiabetische und antiretrovirale Aktivitäten zeigen (Balibar *et al.,* 2007; Bouhired *et al.,* 2007; Fredenhagen *et al.,* 1997; He *et al.,* 2004).

Acetylaszonalenin ist ein Mykotoxin und wurde zusammen mit der nicht-acetylierten Form Aszonalenin aus verschiedenen Pilzen isoliert (Capon *et al.,* 2003; Hayashi *et al.,* 2007; Kimura *et al.,* 1982; Wakana *et al.,* 2006). Roquefortin C ist ebenfalls ein Mykotoxin und wird von *Penicillium roqueforti* und verschiedenen anderen Pilzen produziert (De la Campa *et al.,* 2007; Frisvad & Filtenborg, 1983; Rundberget *et al.,* 2004).

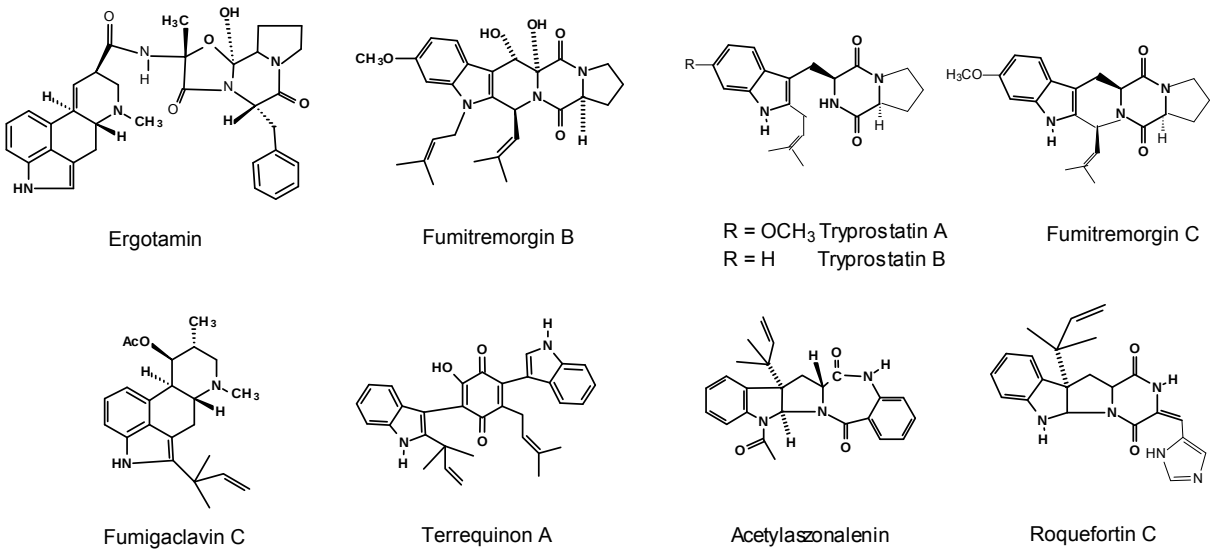

Abb. 1.5: Strukturen einiger prenylierter Indolalkaloide aus Pilzen

1.3 Prenyltransferasen

Prenyltransferasen sind Enzyme, die Isopreneinheiten sowohl miteinander als auch mit anderen Molekülen, wie Proteinen oder Aromaten, verknüpfen können. Sie sind weit verbreitet und kommen im Primär- sowie im Sekundärstoffwechsel vor.

In der Biosynthese von Isoprenoiden werden alle Kohlenstoffgerüste durch *trans*- und *cis*-Prenyltransferasen verknüpft. Die Mitglieder dieser Gruppe von Prenyltransferasen sind sehr gut erforscht. Sie katalysieren die Kondensation von einem C-5 Körper (Isopentenyldiphosphat) mit einem Prenyldiphosphat. *Cis*- und *trans*-Penyltransferasen unterscheiden sich in der Konfiguration ihrer Produkte (Kharel *et al.,* 2006), in ihrer Primärstruktur, aber auch in ihrer dreidimensionalen Struktur voneinander (Fujihashi *et al.,* 2001; Kharel & Koyama, 2003; Shimizu *et al.,* 1998).

Eine weitere Gruppe von Prenyltransferasen sind die sog. Proteinprenyltransferasen. Diese Enzyme katalysieren die Übertragung des C-15 oder C-20 Kohlenstoffgerüst des Farnesyldiphosphates (FPP) bzw. Geranylgeranyldiphosphates (GGPP) auf einen konservierten Cysteinrest in einem CaaX Motif von Protein- oder Peptidsubstraten (Perez-Sala, 2007).

Neben den *trans*- und *cis*- und den Proteinprenyltransferasen ist eine weitere Gruppe von Prenyltransferasen bekannt. Diese Prenyltransferasen werden als sog. aromatische Prenyltransferasen bezeichnet. Die Mitglieder dieser Gruppe katalysieren die Verknüpfung von Prenylresten mit elektronenreichen Aromaten, wie Phenole, Phenolsäuren, Flavonoide, Naphtalene (Heide, 2009), aber auch mit Indolderivaten. Beispiele für zuerst entdeckte aromatische Prenyltransferasen sind UbiA aus *Escherichia coli* (Suvarna *et al.,* 1998; Turunen *et al.,* 2004) und Coq2 aus *Saccharomyces cereviseae* (Ashby *et al.,* 1992). Diese beiden Enzyme sind membrangebundene Proteine und sind in den Primärstoffwechsel involviert. Sie zeigen signifikante Sequenzähnlichkeit zueinander und besitzen ein (N/D)DXXD Motif in ihrer Sequenz. Zu dieser Gruppe von Prenyltransferasen gehören aber auch einige Enzyme des Sekundärstoffwechsels. Beispiele für diese Enzyme sind LePGT-1 und LePGT-2 aus *Lithospermum erythorhizon* (Ohara *et al.,* 2009; Yazaki *et al.,* 2002), HPT aus *Arabidopsis thaliana* (Sadre *et al.,* 2006), OsPPT1c aus *Oryza sativa* (Ohara *et al.,* 2006), SfN8DT-1 aus *Sophora flavescens* (Sasaki et al., 2008) und G4DT aus *Glycine max* (Akashi *et al.,* 2009).

Mittlerweile sind auch einige lösliche Prenyltransferasen aus Bakterien identifiziert worden. Beispiele hierfür sind CloQ aus *Streptomyces roseochromogenes* (Pojer *et al.,* 2003), NaphB aus *Streptomyces* sp CL 190 und Fnq26 aus *Streptomyces cinnamonensis* (Haagen *et al.,* 2007; Kuzuyama *et al.,* 2005; Saleh *et al.,* 2009). Diese löslichen Prenyltransferasen bilden die eigene Gruppe der sog. ABBA-Prenyltransferasen. Die Mitglieder dieser Gruppe zeigen weder Sequenzähnlichkeiten zu *trans*-Prenyltransferasen, noch zu membrangebundenen, aromatischen Prenyltransferasen (Tello *et al.,* 2008). Keine dieser Prenyltransferasen besitzt ein (N/D)DXXD Motif (Saleh *et al.,* 2009).

Lösliche Prenyltransferasen wurden auch in Pilzen entdeckt. Diese Prenyltransferasen unterscheiden sich auf Sequenzebene deutlich von den *cis*- und *trans*-Prenyltransferasen, den membrangebundenen Prenyltransferasen und von den löslichen ABBA-Prenyltransferasen aus Bakterien. Diese löslichen Prenyltransferasen aus Pilzen werden als Indolprenyltransferasen bezeichnet (Steffan *et al.,* 2009a). Indolprenyltransferasen besitzen zwar auch kein (N/D)DXXD Motif in ihrer Sequenz und sind metallionenunabhäng, sie zeigen aber keine Sequenzhomologien zu anderen aromatischen Prenyltransferasen. Ein Enzym aus dieser Gruppe ist eine 4-Dimethylallyltryptophansynthase (4-DMATS). 4-DMATS

prenyliert L-Tryptophan an Position C-4 des Indolrings und katalysiert den ersten Schritt in der Biosynthese von Ergotalkaloiden (Schardl *et al.,* 2006; Tudzynski *et al.,* 2001; Unsöld & Li 2005). Dieses Enzym wurde erstmalig 1971 für *Claviceps* beschrieben (Heinstein *et al.,* 1971), das zugehörige Gen *dmaW* wurde später durch TSAI ET AL. identifiziert (Tsai *et al.,* 1995).

Durch die in den letzten Jahren erhöhte Verfügbarkeit von Genomsequenzen aus Bakterien und Pilzen (Jones, 2007; Raskin *et al.,* 2006) konnten immer mehr Gene aus der Biosynthese von Sekundärmetaboliten identifiziert werden. Für die Gattung *Aspergillus* konnten so bereits eine Reihe von möglichen Prenyltransferasegenen identifiziert werden. Tabelle 1.1 zeigt die Verteilung der entdeckten Prenyltransferasegene auf die unterschiedlichen *Aspergillus*-Stämme (Li, 2009b). Alle diese Prenyltransferasen zeigen auf dem Aminosäurelevel deutliche Sequenzähnlichkeiten zu DMATS aus *Claviceps*.

Tab. 1.1 Identifizierte putative Indolprenyltransferasegene aus verschiedenen *Aspergillus*-Stämmen

Aspergillus-Stämme	Anzahl der putativen Prenyltransferasegene
Aspergillus fumigatus Af293	7
Aspergillus fumigatus A1163	7
Aspergillus nidulans FGSC A4	6
Neosartorya fischeri NRRL181	10
Aspergillus oryzae RIB40	7
Aspergillus niger CBS513.88	2
Aspergillus clavatus NRRL1	3
Aspergillus terreus NIH 2624	10
Aspergillus terreus ATCC 20542	1
Aspergillus flavus NRRL3357	10

Verschiedene Prenyltransferasen aus unterschiedlichen *Aspergillus*-Stämmen sind bereits charakterisiert worden. Die einzelnen Prenyltransferasen unterscheiden sich einerseits durch ihre aromatischen Substrate, aber andererseits auch durch die katalysierten Prenyltransferreaktionen. Bis jetzt sind vier verschiedene

Prenyltransferreaktionen auf unterschiedliche Positionen des Indolrings bekannt: reguläre und reverse C-Prenylierung sowie reguläre und reverse N-Prenylierung (Steffan *et al.,* 2009a). Reguläre Prenylierung bezeichnet die Verknüpfung des Prenylrestes über C-1, wohingegen bei reverser Prenylierung die Bindung über C-3 des Prenyldiphosphates erfolgt (Abb. 1.6).

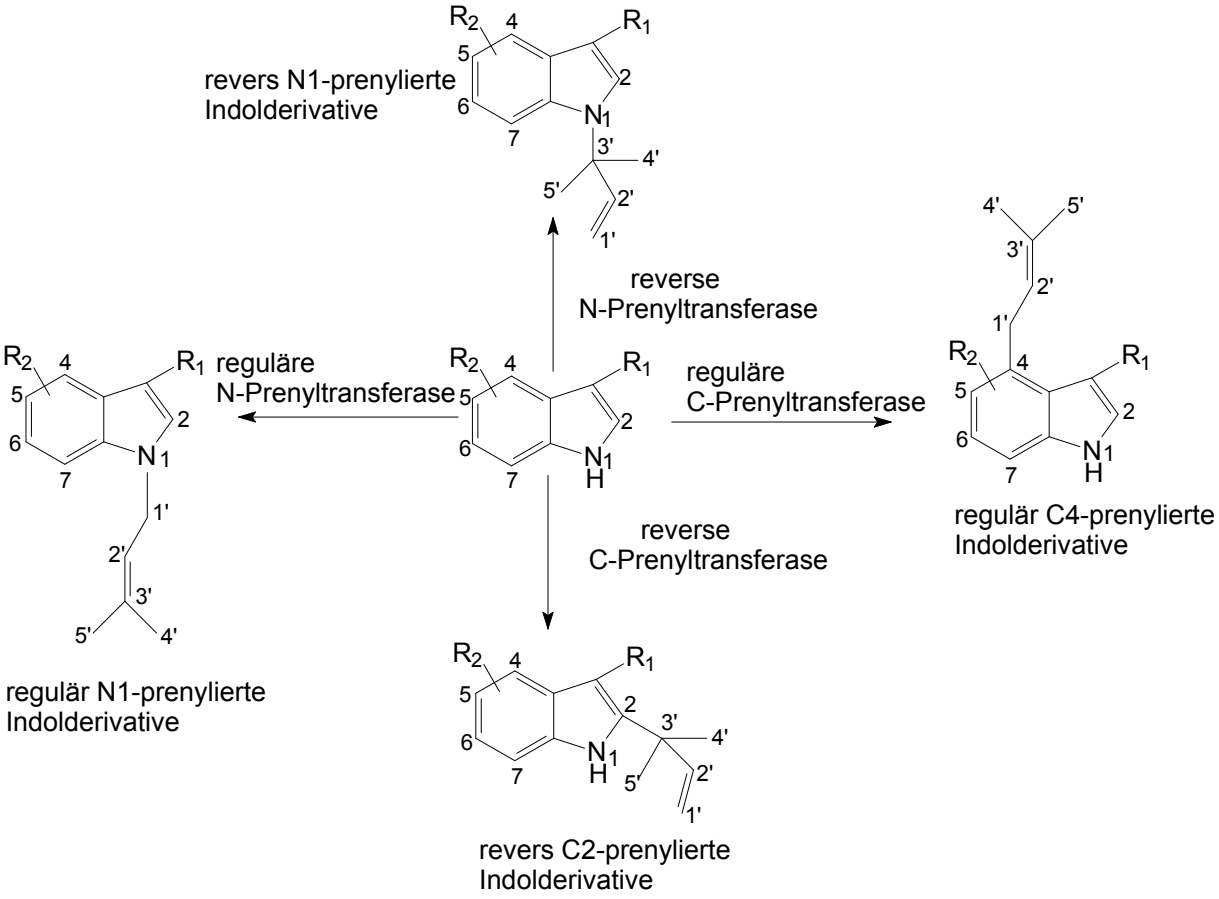

Abb. 1.6: Beispiele für Prenyltransferreaktionen katalysiert durch verschiedene Indolprenyltransferasen (Li 2009a)

Beispiele für Prenyltransferasen, die reguläre C-Prenylierung katalysieren, sind FtmPT1, 7-DMATS, FgaPT2, MaPT und DmaW-Cs (Ding *et al.,* 2008; Grundmann & Li 2005; Kremer *et al.,* 2007; Markert *et al.,* 2008; Unsöld & Li 2005). FgaPT1 aus der Biosynthese von Fumigaclavin C katalysiert die reverse C-Prenylierung von Fumigaclavin A (Unsöld & Li 2006). Beispiele für N-Prenyltransferasen sind FtmPT2 und CdpNPT aus *A. fumigatus*, wobei FtmPT2 die reguläre Prenylierung von 12,13-Dihydroxyfumitremorgin C (Grundmann *et al.,* 2008) und CdpNPT die reverse Prenylierung von tryptophanhaltigen zyklischen Dipeptiden katalysiert (Ruan *et al.,* 2008; Yin *et al.,* 2007).

Von FgaPT2 wurde 2009 durch METZGER ET AL. die dreidimensionale Struktur aufgeklärt. Ein Strukturvergleich von FgaPT2 mit der bakteriellen Prenyltransferase NphB zeigte erstaunlicherweise signifikante Ähnlichkeiten. Auch wenn keine Homologien zwischen der Primärstruktur von aromatischen Indolprenyltransferasen aus Pilzen und bakteriellen Prenyltransferasen der ABBA-Gruppe vorhanden sind, konnte durch die Kristallisierung von FgaPT2 eine strukturelle Ähnlichkeit zwischen FgaPT2 und bakteriellen Prenyltransferasen dieser Klasse gezeigt werden (Metzger *et al.,* 2009).

1.3.1 Chemoenzymatische Synthese von prenylierten Indolalkaloiden

Etwa 75 % der klinisch verwendeten Substanzen sind Naturstoffe oder Derivate von diesen (Wilkinson & Micklefield, 2007). Für die Optimierung von Medikamenten ist es oft notwendig, natürliche Produkte strukturell zu verändern (Walsh, 2003). In den letzten Jahren konnten solche Modifikationen durch verschiedene Methoden, wie kombinatorische Biosynthese, Mutasynthese oder chemoenzymatische Synthese, gesteigert werden. Kombinatorische Biosynthese ist definiert als die Anwendung von genetischen Verfahren zur Modifizierung von Biosynthesewegen von Naturstoffen, um neue oder veränderte Substanzen zu erhalten (Floss, 2006). Mutasynthese hingegen kombiniert genetische mit synthetischen Ansätzen. Diese Methode wurde bereits erfolgreich bei bakteriellen Systemen zur Produktion von biologisch aktiven Substanzen angewendet (Galm *et al.,* 2004; Levengood *et al.,* 2009; Weissman 2007; Weist & Süssmuth 2005). Chemoenzymatische Synthese bedient sich *in vitro* der Hilfe von aufgereinigten Proteinen und wurde schon für die Synthese von neuen bioaktiven Substanzen (Macone *et al.,* 2009), Peptiden (Grunewald & Marahiel 2006; Watanabe *et al.,* 2009), Glykopeptiden (Buskas *et al.,* 2006; Wang 2008), Nukleotiddeoxyzuckern (Rupprath *et al.,* 2005), Glykosiden (Langenhan *et al.,* 2005; Zeng *et al.,* 2007), aber auch für die Entwicklung von neuen Therapeutika (Ran *et al.,* 2009), eingesetzt.

Untersuchungen zur Substratspezifität von Indolprenyltransferasen haben gezeigt, dass alle Enzyme dieser Gruppe spezifisch für DMAPP als Prenyldonor sind (Li 2009). Im Gegensatz dazu zeigen diese Prenyltransferasen beträchtliche Flexibilität gegenüber ihren aromatischen Substraten. FgaPT2 aus der Biosynthese von Fumigaclavin C prenyliert nicht nur Tryptophan, sondern auch verschiedene,

einfache Indolderivate (Steffan *et al.,* 2007) und zyklische Dipeptide, die mindestens eine Typtophaneinheit enthalten (Steffan & Li, 2009). FgaPT2 ist somit ein wichtiges Hilfsmittel für die Produktion von neuen, prenylierten Substanzen. Das Phänomen der breiten Substratspezifität ist jedoch nicht nur auf FgaPT2 begrenzt. Auch eine weitere, in dieser Dissertation bearbeitete Dimethylallyltryptophansynthase, 7-DMATS, akzeptiert eine Vielzahl von aromatischen Substraten und prenyliert diese regiospezifisch an Position C-7 des Indolrings (Kremer & Li, 2008a). Die beiden Prenyltransferasen FtmPT1 und CdpNPT, die als reguläre und reverse zyklische Dipeptidprenyltransferasen charakterisiert wurden (Grundmann & Li 2005; Yin *et al.,* 2007), akzeptieren auch Tryptophan und einfache Indolderivate. Erstaunlicherweise wurden die enzymatischen Produkte der beiden Enzyme als revers prenylierte Indolderivate identifiziert (Zou *et al.,* 2009).

Mit Hilfe der oben aufgeführten Prenyltransferasen ist es möglich, eine Vielzahl von prenylierten Substanzen herzustellen. Diese Enzyme sind somit ein Werkzeug mit großem Potential für die chemoenzymatische Synthese von verschiedenen Substanzen.

1.4 Peptidasen

1.4.1 Allgemeines

Peptidasen bezeichnen eine komplexe Gruppe von Enzymen, die die hydrolytische Spaltung von Peptidbindungen in Peptiden und Proteinen katalysieren. Einer Konvention folgend werden Moleküle aus 100 oder weniger Aminosäuren als Peptide, Moleküle mit mehr als 100 Aminosäuen als Proteine bezeichnet (Löffler, 2005). Peptidasen spalten sowohl Peptide als auch Proteine, wobei Peptidasen, die nur Proteine spalten, zumeist als Proteasen bezeichnet werden. Einige Peptidasen bauen Peptide in ihre konstitutiven Aminosäuren ab, wohingegen andere in posttranslationale Modifikationen involviert sind und selektiv die Spaltung von Peptiden katalysieren (Page & Di, 2008).

Peptidasen spielen eine wichtige Rolle in vielen physiologischen und pathologischen Prozessen. Sie sind involviert in den Proteinkatabolismus, in die Blutkoagluation, in das Zellwachstum, in der Anordnung von Geweben und in das Tumorwachstum.

Peptidasen spielen weiterhin eine Rolle in der Aktivierung von Hormonen und pharmazeutisch aktiven Peptiden durch Spaltung von inaktiven Vorstufen sowie in dem Transport von sekretorischen Proteinen durch Membranen (da Silva *et al.,* 2006). Aufgrund des Einflusses von Peptidasen in verschiedene Prozesse besitzen diese Enzyme ein hohes Potential für die Entwicklung von neuen Medikamenten (Hube 2000; Monod *et al.,* 2002; Rao *et al.,* 1998).

1.4.2 Klassifizierung

Peptidasen werden in Endo- und Exopeptidasen eingeteilt. Endopeptidasen spalten Peptidbindungen innerhalb eines Peptids oder Proteins und werden in vier verschiedene Klassen eingeordnet: Aspartat-, Cystein-, Serin- und Metallo-Peptidasen. Aspartatpeptidasen zeichnen sich durch das Vorkommen von Asparaginsäureresten in ihrem aktiven Zentrum aus. Im Gegensatz dazu besitzen Cysteinpeptidasen nicht nur Cystein, sondern zusätzlich auch noch Histidin in ihrem aktiven Zentrum. Serinpeptidasen sind durch eine Seringruppe in dem aktiven Zentrum charakterisiert. Metallopeptidasen benötigen für eine enzymatische Aktivität zwingend zweiwertige Metallionen (Rao *et al.,* 1998). Im Gegensatz zu Endopeptidasen spalten Exopeptidasen Peptidbindungen entweder vom N- oder C-Terminus aus und gliedern sich in Amino- und Carboxypeptidasen.

Peptidasen werden weiterhin nach ihrer evulotionären Abstammung kategorisiert. Diese Daten können über die MEROPS-Datenbank abgerufen werden (Rawlings & Barrett 1999). In dieser Datenbank werden ähnliche Aminosäuresequenzen als homolog eingestuft und in Familien zusammengefasst. Sie ähneln sich in ihrer Tertiär- bzw. Quartärstruktur. Diese Familien werden außerdem, wenn sie sich aus einem Ur-Protein entwickelt haben, in einen Clan eingebunden, der nach dem Prototyp-Enzym benannt ist (Rawlings & Barrett, 1999).

1.4.2.1 Aminopeptidasen

Aminopeptidasen gehören zu den am längsten bekannten Peptidasen und katalysieren die Aminosäureabspaltung von dem freien Aminoende der verschiedenen Peptide oder Proteine aus. Sie können dabei entweder nur einen Aminosäurerest, ein Dipeptid oder ein Tripeptid abspalten. Manche Aminopeptidasen spalten dabei die Peptidbindung in der Nähe von spezifischen Aminosäureresten,

wohingegen andere die Peptidbindung unspezifisch spalten (Rao *et al.,* 1998). Es sind viele mikrobielle Aminopeptidasen, sowohl aus Bakterien (Addlagatta *et al.,* 2006; Rossier *et al.,* 2008) als auch aus Pilzen (Jalving *et al.,* 2005; Mahon *et al.,* 2009), bekannt. Bei den meisten dieser Enzyme handelt es sich um intrazelluläre Enzyme. Es gibt aber auch Beispiele für extrazelluläre Aminopeptidasen aus Bakterien (Cahan *et al.,* 2007) sowie aus filamentöse Pilzen (Nampoothiri *et al.,* 2005). Sie sind essentiell für verschiedene Stoffwechselwege, u. a. für den Abbau von nichthormonellen und hormonellen Peptiden und für posttranslationale Modifikationen von Proteinen (Taylor, 1993). Die Mehrheit der Aminopeptidasen gehört zu der Klasse der Metalloenzyme. Sie benötigen Zink für ihre Aktivität und besitzen ein hochkonserviertes Zinkbindungsmotif HEXXH (Hooper, 1994; Jongeneel *et al.,* 1989). Es sind jedoch auch Aminopeptidasen bekannt, die dieses Motif nicht besitzen (Golich *et al.,* 2006). Die Klassifizierung erfolgt in der Regel auf Grundlage der Substratspezifität und auf dem Verhalten gegenüber bestimmten Inhibitoren.

2 Zielsetzung der Arbeit

Die erste Zielsetzung meiner Arbeit war die biochemische Charakterisierung der Indolprenyltransferase 7-DMATS aus A. *fumigatus*. Dafür mussten verschiedene Arbeiten durchgeführt werden:

- Sequenzanalyse des putativen Prenyltransferasegens *Afu3g12930* aus *A. fumigatus* Af293
- Sequenzvergleich mit anderen Prenyltransferasen
- Klonierung von *Afu3g12930* (diese Arbeit wurde von Frau Lucia Westrich durchgeführt) und Überproduktion und Aufreinigung von His_6-7-DMATS
- biochemische Charakterisierung von 7-DMATS

Im Zuge der Untersuchungen zur Substratspezifität von 7-DMATS stellte sich die Frage, inwieweit 7-DMATS verschiedene Indolderivate akzeptiert und an welcher Position des Indolringes die Prenylierung stattfindet. Weiterhin stellte sich die Frage, ob sich dieses Enzym für eine chemoenzymatische Synthese eignet. Zur Klärung dieser Fragestellungen wurden weitere Arbeiten notwendig:

- Test von verschiedenen Indolderivaten und anschließende Strukturaufklärung der enzymatischen Produkte
- Versuche zur Steigerung der Umsetzungsraten von verschiedenen Indolderivaten

Durch die Versuche zur Substratspezifität von 7-DMATS konnte eine katalytische Promiskuität dieses Enzyms festgestellt werden. Dadurch ergab sich der zweite Teil meiner Arbeit, die Charakterisierung dieser zweiten enzymatischen Aktivität. Dazu wurden verschiedene Arbeiten notwendig:

- biochemische Charakterisierung der Peptidaseaktivität von 7-DMATS
- Untersuchungen von weiteren Indolprenyltransferasen aus Pilzen hinsichtlich einer möglichen katalytischen Promiskuität

Der letzte Teil meiner Arbeit hatte zum Ziel, einen Schritt aus der Sirodesminbiosynthese aus *L. maculans* aufzuklären. Dazu mussten folgende Arbeiten durchgeführt werden:

- Klonierung von *sirD* und Überproduktion und Aufreinigung von His_6-SirD
- biochemische Charakterisierung von SirD

3 Ergebnisse und Diskussion

Dieser Teil der Dissertation enthält eine Zusammenfassung der Veröffentlichungen, die im Verlauf der Promotionszeit entstanden sind. Für weiterführende Informationen sind diese Veröffentlichungen der Dissertation im Anhang beigefügt.

3.1 Überproduktion, Aufreinigung und biochemische Charakterisierung einer 7-Dimethylallyltryptophansynthase aus *Aspergillus fumigatus*

Aus dem Genom von *A. fumigatus* Af293 wurden bis jetzt 26 Gencluster, die für verschiedene Sekundärmetabolite kodieren, identifiziert (Nierman *et al.,* 2005). Eines dieser putativen Cluster besteht aus vermutlich acht Genen und kodiert möglicherweise für einen noch unbekannten Metaboliten. Dieses Cluster enthält drei Gene mit signifikanten Sequenzhomologien zu Genen sowohl aus dem Gliotoxinbiosynthesecluster von *A. fumigatus* (Gardiner & Howlett 2005) als auch zu Genen aus dem Sirodesminbiosynthesecluster von *L. maculans* (Gardiner et al., 2004). Bei beiden Substanzen handelt es sich um Epipolythiodioxopiperazine. Von den acht Genen dieses putativen Biosyntheseclusters kodieren zwei Gene für putative Transkriptionsfaktoren und ein Gen kodiert für einen putativen Transporter. Weiterhin konnte ein Gen für eine nicht-ribosomale Peptidsynthetase, ein Gen für eine putative Methyltransferase sowie ein Gen für ein putatives P-450 Enzym identifiziert werden. Dieses Cluster enthält jedoch kein Gen, das für eine Thiodoxinreduktase kodiert. Diese Reduktase ist essentiell für die Bildung der Disulfidbindung in Epipolythiodioxopiperazinen. Es kann somit spekuliert werden, dass das Produkt dieses Clusters, trotz der Homologien zu den Clustern von Gliotoxin und Sirodesmin, nicht für ein Epipolythiodioxopiperazin kodiert oder dass dieses Cluster unvollständig ist. Gegen die Vermutung des unvollständigen Clusters spricht, dass das Cluster auch in *Neosartorya fischeri* NRRL181 und in *Aspergillus terreus* NIH2624 vorhanden ist. Durch Homologievergleiche konnte in diesem Cluster zusätzlich ein putatives Prenyltransferasegen, *Afu3g12930*, identifiziert werden. *Afu3g12930* enthält zwei Exons mit einer Größe von 1301 und 118 Basenpaaren und ist unterbrochen durch ein Intron von 53 Basenpaaren. Das Genprodukt, EAL92290 (7-DMATS), umfasst 472 Aminosäuren und besitzt eine berechnete Größe von 53

kDa. 7-DMATS zeigt eine Sequenzähnlichkeit von 34 % zu der Prenyltransferase SirD aus der Biosynthese von Sirodesmin PL in *L. maculans* und 31 % zu einer Dimethylallyltryptophansynthase (FgaPT2) aus *A. fumigatus*. Eine Sequenzhomolgie von 28 % zu FtmPT1 aus dem Fumitremorginbiosynthesecluster und zu FgaPT1 aus dem Fumigaclavinbiosynthesecluster konnte bestimmt werden.

Für die Charakterisierung von 7-DMATS wurde die kodierende Sequenz von *Afu3g12930* durch L. Westrich in den Expressionsvektor pQE60 kloniert und in *E. coli* überexprimiert. Das lösliche His_6-Fusionsprotein wurde im Zuge dieser Dissertation mit Hilfe von Ni-NTA Agarose bis zur Homogenität aufgereinigt, dabei konnte eine Proteinmenge von 5 mg pro Liter Kultur erreicht werden. Für einen Nachweis der enzymatischen Aktivität wurde 7-DMATS in Anwesenheit des Prenyldonors Dimethylallyldiphosphat (DMAPP) mit verschiedenen aromatischen Substraten inkubiert. HPLC-Analysen dieser Inkubationsansätze zeigten bei verschiedenen Substraten eine Produktbildung, die in Kontrollansätzen mit hitze-denaturiertem Enzym nicht zu beobachten war. Bei diesen Versuchen konnte L-Tryptophan als Substrat mit der höchsten Umsetzungsrate bestimmt werden. Dies war ein Indiz dafür, dass L-Tryptophan das natürliche Substrat von 7-DMATS ist. Die Struktur des enzymatischen Produktes konnte mit Hilfe von LC-MS- und NMR-Analysen eindeutig als Tryptophan mit einem Prenylrest an Position C-7 des Indolrings identifiziert werden.

Die Prenylierung von L-Tryptophan war strikt abhängig von der Anwesenheit des Prenyldonors Dimethylallyldiphosphat (DMAPP). Bei Verwendung von Geranyldiphosphat (GPP) anstelle von DMAPP konnte keine Produktbildung beobachtet werden. Im Gegensatz dazu zeigte 7-DMATS eine relativ hohe Flexibilität bezüglich des aromatischen Substrates. Es wurden sowohl verschiedene tryptophanhaltige zyklische Dipeptide als auch verschiedene Indolderivate umgesetzt. Andere aromatische Aminosäuren wie Phenylalanin oder Tyrosin wurden nicht prenyliert.

Neben Versuchen zur Substratspezifität wurden die Ionenabhängigkeit und der K_M-Wert von 7-DMATS bestimmt. 7-DMATS erwies sich als unabhängig von divalenten Metallkationen. Trotz Zugabe von EDTA, einem Komplexbildner für zweiwertige Metallionen, konnte keine Verringerung der enzymatischen Aktivität festgestellt werden. Dieses Ergebnis entsprach den Ergebnissen für andere

Indolprenyltransferasen. Die enzymatische Reaktion von 7-DMATS folgte der Michaelis-Menten Kinetik, K_M-Werte von 137 µM für L-Tryptophan und 67 µM für DMAPP konnten bestimmt werden.

Für eine Zusammenfassung der Ergebnisse wird auf folgende Publikation verwiesen:
Kremer, A., Westrich, L. and Li, S.-M. (2007)
A 7-dimethylallyltryptophan synthase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterization
Microbiology 153, 3409-3416

3.2 Das Potential der 7-DMATS als Werkzeug für die Produktion von prenylierten Indolderivaten

Die Grundlage dieser Arbeit wurde durch die biochemische Charakterisierung der 7-Dimethylallyltryptophansynthase (7-DMATS) aus *A. fumigatus* gelegt. In dieser ersten Studie konnten Hinweise auf eine breite Substratspezifität von 7-DMATS festgestellt werden. In der vorliegenden Studie wurden weitere Versuche zur Substratspezifität von 7-DMATS durchgeführt. Insgesamt wurde die Akzeptanz von 24 einfachen Indolderivaten getestet.

Für eine bessere Übersicht wurden die 24 getesteten Indolderivate in zwei verschiedene Gruppen eingeteilt. Die erste Gruppe umfasste alle Indolderivate mit Modifikationen an der Seitenkette, d. h. mit unterschiedlichen Substitutionen an Position C-3 des Indolrings. Die zweite Gruppe umfasste Indolderivate mit verschiedenen Modifikationen an unterschiedlichen Positionen des Indolrings.

Es konnte gezeigt werden, dass 7-DMATS, bis auf eine Ausnahme im Falle von 7-Methyltryptophan, alle getesteten Indolderivate akzeptiert. Die enzymatischen Produkte konnten mit Hilfe von LC-MS Analytik eindeutig als einfach prenylierte Substanzen identifiziert werden.

Die 14 Mitglieder der ersten Gruppe wurden von 7-DMATS unterschiedlich gut umgesetzt. Die Umsetzungsraten lagen dabei zwischen 4 und 89 %. Eine Erkenntnis aus diesen Versuchen ist der signifikante Einfluss der Konformität an Position C-11 auf die Umsetzungsrate. Bei Verwendung von D- anstelle von L-Tryptophan konnte

nur 11,8 % der Umsetzungsrate von L-Tryptophan erreicht werden. Modifikationen an der Seitenkette bewirkte in jedem Fall eine Reduktion der Umsetzungsrate. Desaminierung des Tryptophans zu Indol-3-Propionsäure z. B. bewirkte eine Reduktion der Umsetzungsrate auf 8,5 %. Vergleichbare Werte konnten für Indol-3-Essigsäure und Indol-3-Buttersäure bestimmt werden. Decarboxylierung von Tryptophan zu Tryptamin, Methylierung zu L-Tryptophan-Methylester oder Acetylierung zu N-Acetyltryptophan führte ebenfalls zu einer deutlich reduzierten, aber immer noch nachweisbaren enzymatischen Aktivität von 7-DMATS. Im Gegensatz dazu konnten höhere Umsetzungsraten von 82,2 und 28,2 % für L-Abrin und L-β-Homotryptophan beobachtet werden.

Von den 10 Mitgliedern der zweiten Gruppe wurden 9 Substanzen akzeptiert. Eine Ausnahme bildete nur 7-Methyltryptophan. Diese Ausnahme bestätigte die Regioselektivität der enzymatischen Reaktion, da bei 7-Methyltryptophan der Ort der Prenylierung durch eine Methylgruppe blockiert ist. Eine Methylierung am Indolring führte zu einer Reduktion der Umsetzungsrate, wobei der Abstand der Methylgruppe von C-7, dem Ort der Prenylierung, einen deutlichen Einfluss auf die Umsetzungsrate hatte. Je näher der Prenylrest an diese Position gebunden war, umso höher war der negative Einfluss auf die enzymatische Aktivität von 7-DMATS. So war z. B. die Umsetzungsrate von 6-Methyltryptophan mit 19,8 % deutlich geringer als die Umsetzungsrate von 4-Methyltryptophan mit 89,4 %. Auch Halogenierung des Indolrings bewirkte nur eine Reduktion der Umsetzungsrate, nicht aber den vollständigen Verlust der Akzeptanz durch 7-DMATS.

Für die Bestätigung der angenommen Prenylierungsposition an C-7 des Indolrings der verschiedenen Indolderivate, wurden die Strukturen von 10 prenylierten Indolderivaten mit Hilfe von NMR-Analytik aufgeklärt. In allen Fällen konnte gezeigt werden, dass auch Indolderivate regioselektiv an Position C-7 prenyliert werden.

Aufgrund der oben dargestellten breiten Substratspezifität von 7-DMATS bot sich diese Prenyltransferase als ein Werkzeug für die chemoenzymatische Synthese von prenylierten Indolderivaten an. Unter verbesserten Bedingungen, d. h. durch eine Erhöhung der eingesetzten Enzymmenge und durch Verlängerung der Inkubationszeit konnte bei L-Tryptophan, L-Abrin und L-β-Homotryptophan eine nahezu quantitative Umsetzung in das prenylierte Produkt erreicht werden. Ebenfalls sehr hohe Umsetzungsraten zwischen 84 und 95 % konnten für 4-Methyltryptophan,

5-Methyltryptophan und für 11-Methyltryptophan erreicht werden. Etwas geringere, aber immer noch Umsetzungsraten von über 50 % konnten für 6-Methyltryptophan und 6-Fluorotryptophan beobachtet werden.

Für eine Zusammenfassung der Ergebnisse wird auf folgende Publikation verwiesen:
Kremer, A. and Li, S.-M. (2008)
Potential of a 7-dimethylallyltryptophan synthase as a tool for production of prenylated indole derivatives
Appl. Microbiol. Biotechnol. 79, 951-961

3.3 Tryptophanaminopeptidaseaktivität von verschiedenen Indolprenyltransferasen aus *A. fumigatus*

Die Prenyltransferasen 7-DMATS, FgaPT1, CdpNPT und FtmPT1 sind in verschiedenste Biosynthesewege involviert und katalysieren die Prenylierung an unterschiedlichen Positionen des Indolrings. Sie wurden bereits in verschiedenen Studien biochemisch charakterisiert (Grundmann & Li 2005; Kremer *et al.,* 2007; Ruan *et al.,* 2008; Unsöld & Li 2006; Yin *et al.,* 2007). FgaPT1 ist in die Biosynthese von Fumigaclavin C involviert und katalysiert die Prenylierung an Position C-2 des Indolrings von Fumigaclavin A. FtmPT1 prenyliert an der gleichen Position, ist aber ein Enzym in der Biosynthese von Fumitremorgin B und prenyliert das zyklische Dipeptid Brevianamid F. Für die beiden Prenyltransferasen CdpNPT und 7-DMATS konnte bis jetzt noch kein Biosyntheseweg identifiziert werden. In beiden Fällen konnte jedoch eine enzymatische Aktivität eindeutig nachgewiesen werden. CdpNPT katalysiert die Prenylierung von zyklischen Dipeptiden an Position C-1, wohingegen 7-DMATS als Dimethylallyltryptophansynthase fungiert und Tryptophan an Position C-7 des Indolrings prenyliert.

Im Zuge dieser Dissertation wurde zum ersten Mal die katalytische Promiskuität dieser Prenyltransferasen gezeigt. Alle vier Prenyltransferasen katalysieren neben der Prenyltransferasereaktion auch die Hydrolyse von linearen Peptiden. Untersuchungen zur Substratspezifität dieser Peptidaseaktivitäten zeigten, dass die Prenyltransferasen verschiedene tryptophanhaltige Dipeptide akzeptieren. Bei diesen

Dipeptiden musste aber immer die Aminogruppe des Tryptophans frei vorliegen. Dipeptide mit einer gebundenen Tryptophanaminogruppe wurden in keinem Fall akzeptiert. Basierend auf der Bedingung der ungebundenen Aminogruppe resultierte die Schlussfolgerung, dass es sich bei der festgestellten Peptidaseaktivität um eine Tryptophanaminopeptidaseaktivität handelt.

Es konnte gezeigt werden, dass die Tryptophanaminopeptidaseaktivitäten der vier Prenyltransferasen sich in Bezug auf die Ausprägung dieser Aktivität deutlich voneinander unterscheideten. Eine Übernachtinkubation von 3 µg 7-DMATS mit dem linearen Dipeptid aus L-Tryptophan und Glycin (H-L-Trp-Gly-OH) führte zu einer nahezu quantitativen Hydrolyse des Dipeptides. Für FgaPT1 konnte unter den gleichen Bedingungen nur eine Umsetzungsrate von 63 % bestimmt werden. Noch deutlicher ist der Unterschied zwischen 7-DMATS und CdpNPT bzw. FtmPT1. Bei diesen Prenyltransferasen konnten Umsetzungsraten von nur 18 bzw. 10 % beobachtet werden.

Untersuchungen zum Einfluss von Metallionen auf die Peptidaseaktivität zeigten einen weiteren Unterschied der verschiedenen Prenyltransferasen. Die Zugabe von Mn^{2+} Ionen bewirkte bei 7-DMATS und FgaPT1 eine leichte Hemmung auf 84 bzw. 70 %, wohingegen die Zugabe der gleichen Ionen eine deutliche Steigerung der Peptidaseaktivität von FtmPT1 und CdpNPT auf das 4 bzw. 6 fache bewirkte. In allen vier Fällen wurde die Peptidaseaktivität durch Zugabe des metallchelatierenden Agens EDTA deutlich gehemmt. Diese Hemmung verdeutlicht die strikte Metallionenabhängigkeit der Peptidaseaktivität der Prenyltransferasen.

Für ein besseres Verständnis dieser neuen Enzymgruppe wurden verschiedene kinetische Parameter bestimmt und mit den Parametern der Prenyltransferaseaktivitäten verglichen. Auch die Peptidaseaktivitäten folgten der Michaelis-Menten Kinetik, wobei alle bestimmten K_M-Werte signifikant höher und die Wechselzahlen signifikant niedriger waren als die Werte der zugehörigen Prenyltransferaseaktivität. Weiterhin zeigte ein Vergleich der K_M-Werte der Prenyltransferaseaktivität mit den K_M-Werten der Peptidaseaktivität deutlich, dass die K_M-Werte der Peptidaseaktivität der einzelnen Prenyltransferasen sehr viel geringere Abweichungen zueinander hatten und alle zwischen 0,3 und 0,42 mM lagen.

Es konnte nicht bestimmt werden, ob oder welche Rolle die Peptidaseaktivitäten in der Biosynthese der zugehörigen Sekundärmetabolite spielen und ob sie einen

Einfluss auf die Prenyltransferaseaktivität besitzen. Eine direkte Korrelation zwischen Peptidase- und Prenytransferaseaktivität konnte nicht beobachtet werden.

Für eine Zusammenfassung der Ergebnisse wird auf folgende Publikation verwiesen:
Kremer, A. and Li, S.-M. (2008)
Tryptophan aminopeptidase activity of several indole prenyltransferases from *Aspergillus fumigatus*
Chem. Biol. 15, 729-738

3.4 Neue Erkenntnisse der katalytischen Promiskuität von pilzlichen Indolprenyltransferasen: Metallionen, chelatierende und reduzierende Substanzen beeinflussen ihre Aminopeptidaseaktivität

Nachdem in der vorangegangene Studie bereits die katalytische Promiskuität von vier Indolprenyltransferasen nachgewiesen wurde (Kremer & Li 2008b), wurden in dieser Studie vier weitere Prenyltransferasen hinsichtlich einer möglichen Aminopeptidaseaktivität untersucht. Diese Enzyme stammen aus unterschiedlichen *Aspergillus*-Stämmen und katalysieren verschiedene Prenylierungsreaktionen. Untersucht wurden TdiB aus *A. nidulans*, AnaPT aus *N. fischeri*, sowie FgaPT2 und FtmPT2, beide aus *A. fumigatus*. TdiB ist in die Biosynthese von Terrequinon A involviert und katalysiert die Prenylierung von Didemethylasterriquinon D (Schneider *et al.,* 2008). AnaPT katalysiert die Prenylierung von Benzodiazepinedion in der Biosynthese von Acetylaszonalenin (Yin *et al.,* 2009). FgaPT2 fungiert als Dimethylallyltryptophansynthase in der Fumigaclavinbiosynthese (Unsöld & Li 2005), wohingegen FtmPT2 12,13-Dihydroxyfumitremorgin C prenyliert und in die Biosynthese von Verruculogen involviert ist (Grundmann *et al.,* 2008; Steffan *et al.,* 2009b). Für einen Nachweis der Aminopeptidaseaktivität von FtmPT2, TdiB und AnaPT wurden alle Enzyme mit dem linearen Dipeptid H-L-Trp-Gly-OH für 16 Stunden inkubiert. HPLC-Analysen dieser Inkubationsansätze zeigten in allen drei Fällen eine deutliche Aminopeptidaseaktivität mit Umsetzungsraten von 31, 22 und 45 % für FtmPT2, TdiB und AnaPT. Erstaunlicherweise konnte, wie auch bereits in

der vorherigen Studie, für FgaPT2 keine Aminopeptidaseaktivität beobachtet werden. Dieses Ergebnis war insofern verwunderlich, da alle getesteten Indolprenyltransferasen eine zweite enzymatische Aktivität besitzen. Aus diesem Grund wurden weitere Untersuchungen bezüglich möglicher inhibierender Substanzen auf die Peptidaseaktivität von FgaPT2 durchgeführt. Neben anderen Substanzen wurde Dithiothreitol (DTT) getestet. DTT ist ein reduzierendes Agens und wird u.a. zur Stabilisierung von aufgereinigten Prenyltransferasen verwendet. Ein Beispiel für diese Form der Verwendung ist MaPT aus *Malbranchea aurantiaca* (Ding *et al.,* 2008). Von den getesteten Prenyltransferasen wurde nur FgaPT2 in der Anwesenheit von DTT aufbewahrt. Um auszuschließen, dass DTT die Peptidaseaktivität von FgaPT2 hemmt, wurde DTT aus dem Proteinaufbewahrungspuffer entfernt und FgaPT2 erneut mit H-L-Trp-Gly-OH inkubiert. Die HPLC-Analyse des Inkubationsgemisches zeigte eindeutig die Freisetzung von Tryptophan. Daraus konnte geschlossen werden, dass FgaPT2 auch als Aminopeptidase fungiert. Für die Klärung der Frage, ob diese Inhibierung spezifisch für FgaPT2 ist oder ob dies generell ein Phänomen aller Prenyltransferasen ist, wurden sowohl die Prenyltransferasen aus dieser Studie als auch die Prenyltransferasen 7-DMATS, FgaPT1, CdpNPT und FtmPT1 in Anwesenheit von DTT mit H-L-Trp-Gly-OH inkubiert. Für FgaPT1, FtmPT2, 7-DMATS, AnaPT und TdiB konnte eine komplette Hemmung durch DTT beobachtet werden. Im Gegensatz dazu hatte DTT nur einen geringen Einfluss auf die Aminopeptidaseaktivität von CdpNPT und FtmPT1. In beiden Fällen konnten in Anwesenheit von DTT Umsetzungsraten von 79 bzw. 66 % für CdpNPT bzw. für FtmPT1 beobachtet werden. Die Zugabe von reduziertem Gluthation, einem weiterem reduzierenden Agens, bewirkte in allen Fällen eine komplette Inhibierung der Peptidaseaktivität.

Die in dieser und in der vorangegangenen Studie untersuchten Prenyltransferasen zeigten alle keine Sequenzähnlichkeiten zu bereits bekannten Peptidasen. Für einen Vergleich der biochemischen Eigenschaften mit bekannten Peptidasen wurde der Einfluss der Cysteinproteaseinhibitoren Leupeptin, E-64 und EST (Barrett *et al.,* 1982) und des Chymotrypsinproteaseininhibitors TPCK (Stoppler *et al.,* 1996) auf die Peptidaseaktivität getestet. In keinem Fall konnte ein signifikanter Effekt auf die Aminopeptidaseaktivität der Prenyltransferasen festgestellt werden.

Die Aminopeptidaseaktivitäten der in dieser Studie untersuchten Prenyltransferasen sind alle metallionenabhängig. Inkubationen mit den beiden metallchelatierenden Agenzien EDTA und 1,10-Phenantrolin bewirkte in allen Fällen eine deutliche Reduktion der Peptidaseaktivität. Diese Tatsache entspricht den Ergebnissen von 7-DMATS, FgaPT1, FtmPT1 und CdpNPT.

Vergleichbar zu den Ergebnissen der ersten Studie hatte die Zugabe von Mn^{2+} Ionen keinen einheitlichen Effekt auf die Peptidaseaktivität von FgapT2, FtmPT2, TdiB und AnaPT. Im Fall von FgaPT2 bewirkte die Zugabe von Mn^{2+} eine deutliche Zunahme der Peptidaseaktivität, wohingegen bei FtmPT2, AnaPT und TdiB ein negativer Einfluss zu beobachten war. Diese Zweiteilung der Prenyltransferasen konnte auch schon für 7-DMATS, FgaPT1, FtmPT1 und CdpNPT beobachtet werden. Nur bei CdpNPT und bei FtmPT1 konnte durch Zugabe von Mn^{2+} Ionen eine Steigerung der Aminopeptidaseaktivität beobachtet werden. Das Phänomen des unterschiedlichen Einflusses von Metallionen auf die Peptidaseaktivität könnte entweder darauf basiert sein, dass bei manchen Prenyltransferasen Metallionen stärker im aktiven Zentrum des Enzyms gebunden sind oder darauf, dass für die Katalyse unterschiedliche Metallionen benötigt werden.

Aufgrund des unterschiedlichen Verhaltens der Prenyltransferasen auf die Zugabe von Mn^{2+} Ionen und von DTT könnte spekuliert werden, dass Indolprenyltransferasen aus Pilzen zu verschiedenen Untergruppen gehören. Die erste Untergruppe würde die Prenyltransferasen umfassen, deren Peptidaseaktivität nur sehr gering durch DTT gehemmt werden können. Die Aminopeptidaseaktivität der Mitglieder dieser Untergruppe kann jedoch durch die Zugabe von Mn^{2+} Ionen deutlich gesteigert werden. Aufgrund der aufgestellten Kriterien würden CdpNPT und FtmPT1 dieser Untergruppe angehören. Die zweite Untergruppe würde die Prenyltransferasen umfassen bei denen eine Zugabe von Mn^{2+} Ionen die Peptidaseaktivität senkt. Auch eine Zugabe von DTT führt bei den Mitgliedern dieser Untergruppe zu einer deutlichen Hemmung der Peptidaseaktivität. In diese Untergruppe könnten FtmPT2, TdiB, AnaPT und 7-DMATS eingeordnet werden. FgaPT1 und FgaPT2 vereinen verschiedene Aspekte der beiden anderen Untergruppen und können somit nicht eindeutig einer der beiden Gruppen zugeordnet werden. Aus diesem Grund würden diese beiden Prenyltransferasen eine seperate Untergruppe bilden.

Für eine Zusammenfassung der Ergebnisse wird auf folgende Publikation verwiesen:
Kremer, A., Stec, E., Yin, W.-B., Grundmann, A. and Li, S.-M. (2009)
New insights into the catalytic promiscuity of fungal indole prenyltransferases: metal ions, chelating and reducing agents impact their aminopeptidase activity
in Vorbereitung

3.5 Eine Tyrosin O-Prenyltransferase katalysiert den ersten Schritt in der Biosynthese von Sirodesmin PL

In dem Genom des phytopathogenen Pilz *L. maculans* konnte bereits 2004 ein putatives Gencluster aus 18 Genen, das für die Biosynthese von Sirodesmin PL kodiert, identifiziert werden (Gardiner *et al.,* 2004). Dieses Biosynthesecluster enthält u. a. ein Gen, *sirD*, das für eine putative Prenyltransferase kodiert. SirD ist in eine der ersten Schritte der Biosynthese von Sirodesmin PL involviert, wobei das natürliche Substrat bis jetzt nicht bestimmt werden konnte. Spekuliert wurde zum Einen, das SirD das erste Enzym dieses Biosyntheseweges ist und die Prenylierung von Tyrosin katalysiert aber zum Anderen auch, ob der Biosyntheseweg mit der Bildung des zyklischen Dipeptids aus Tyrosin und Serin beginnt und SirD anschließend das Dipeptid prenyliert. SirD besitzt zudem eine Sequenzähnlichkeit zu 7-DMATS, so dass auch Tryptophan das natürliche Substrat von SirD sein könnte. Für die Klärung dieser Frage wurde die vorliegende Studie durchgeführt.

Das Gen *sirD* enthält zwei Exons mit einer Größe von 1217 und 133 Basenpaaren, unterbrochen durch ein Intron mit einer Größe von 51 Basenpaaren. Das Genprodukt SirD besteht aus 449 Aminosäuren und hat eine berechnete Größe von 51 kDa. SirD besitzt Sequenzhomologien von 34 bzw. 31 % zu den beiden Dimethylallyltryptophansynthasen 7-DMATS und FgaPT2 aus *A. fumigatus.*

Die kodierende Region von *sirD* wurde aus cDNA amplifiziert und in den Expressionsvektor pQE70 kloniert. Anschließend wurde das Gen in *E. coli* heterolog überexprimiert und das His_6-Fusionsprotein aufgereinigt. Eine Proteinausbeute von 2 mg pro Liter Kultur konnte erreicht werden. Zur Klärung der Frage des natürlichen Substrates wurde SirD in Anwesenheit des Prenyldonors DMAPP sowohl mit cyclo-L-Tyr-L-Ser als auch mit L-Tyrosin inkubiert. HPLC-Analysen der Inkubationsgemische

zeigten nur im Inkubationsansatz mit Tyrosin einen Produktpeak. Für eine Strukturbestimmung dieses enzymatischen Produktes wurde der Produktpeak isoliert und mit Hilfe von NMR-Analytik analysiert. Die Isolation wurde in Anwesenheit von säurehaltigem Fließmittel durchgeführt. Das hatte zur Folge, dass das enzymatische Produkt zum großen Teil wieder in das Substrat hydrolysiert wurde. Deutlich wurde dieses Phänomen durch das aufgenommene NMR Spektrum, da hier Signale für zwei Substanzen gefunden werden konnte. Eine Substanz konnte eindeutig als Tyrosin und die andere als prenyliertes Tyrosin identifiziert werden. Die Instabilität des enzymatischen Produktes war ein Indiz für eine Prenylierung an der phenolischen Hydroxylgruppe. Um diese Hypothese zu stützen, wurde das enzymatische Produkt erneut isoliert, es wurde jedoch Fließmittel ohne Säure verwendet. Auf diese Art konnte das Produkt eindeutig als O-prenyliertes Tyrosin identifiziert werden.

Inkubationen von SirD mit Phenylalanin, 4-Hydroxybenzoesäure und *para*-Cumarsäure in Anwesenheit von DMAPP führte zu keiner Produktbildung. Dies zeigte die absolute Notwendigkeit der Hydroxylgruppe an Position C-4 des aromatischen Rings von Tyrosin und der Aminosäurestruktur an der Seitenkette. Eine zusätzliche Hydroxylgruppe im Fall des L-3,4-Dihydroxyphenylalanin bewirkte eine Reduktion der Umsetzungsrate, eine Produktbildung war aber noch deutlich zu detektieren. Erstaunlicherweise akzeptierte SirD nicht nur Tyrosin, sondern auch L-Tryptophan und zeigte somit eine breitere Substratspezifität als andere Prenyltransferasen aus Pilze. Das prenylierte Produkt konnte eindeutig als 7-Dimethylallyltryptophan identifiziert werden.

Durch die duchgeführten Versuche konnte die Frage des natürlichen Substrates von SirD eindeutig geklärt werden. SirD katalysiert die Prenylierung von Tyrosin und fungiert als O Tyrosin-Prenyltransferase und stellt somit das erste Enzym in der Biosynthese von Sirodesmin PL dar.

Zusätzlich konnte gezeigt werden, dass SirD katalytische Promiskuität besitzt und als Aminopeptidase fungiert. Im Gegensatz zu Indolprenyltransferasen akzeptierte SirD als hydrolytisches Substrat dabei nicht nur H-L-Trp-Gly-OH sondern auch H-L-Tyr-Gly-OH. In beiden Fällen konnte bei einer Inkubation über Nacht eine fast vollständige Hydrolyse des Substrates beobachtet werden. Die Ergebnisse dieser Versuche sind in Abb. 1.7 dargestellt. SirD benötigte für die Hydrolyse des linearen

Dipeptides aus Tyrosin und Glycin eine freie Aminogruppe von Tyrosin. Eine Inkubation von SirD mit H-Gly-L-Tyr-OH führte zu keiner Hydrolyse des Substrates.

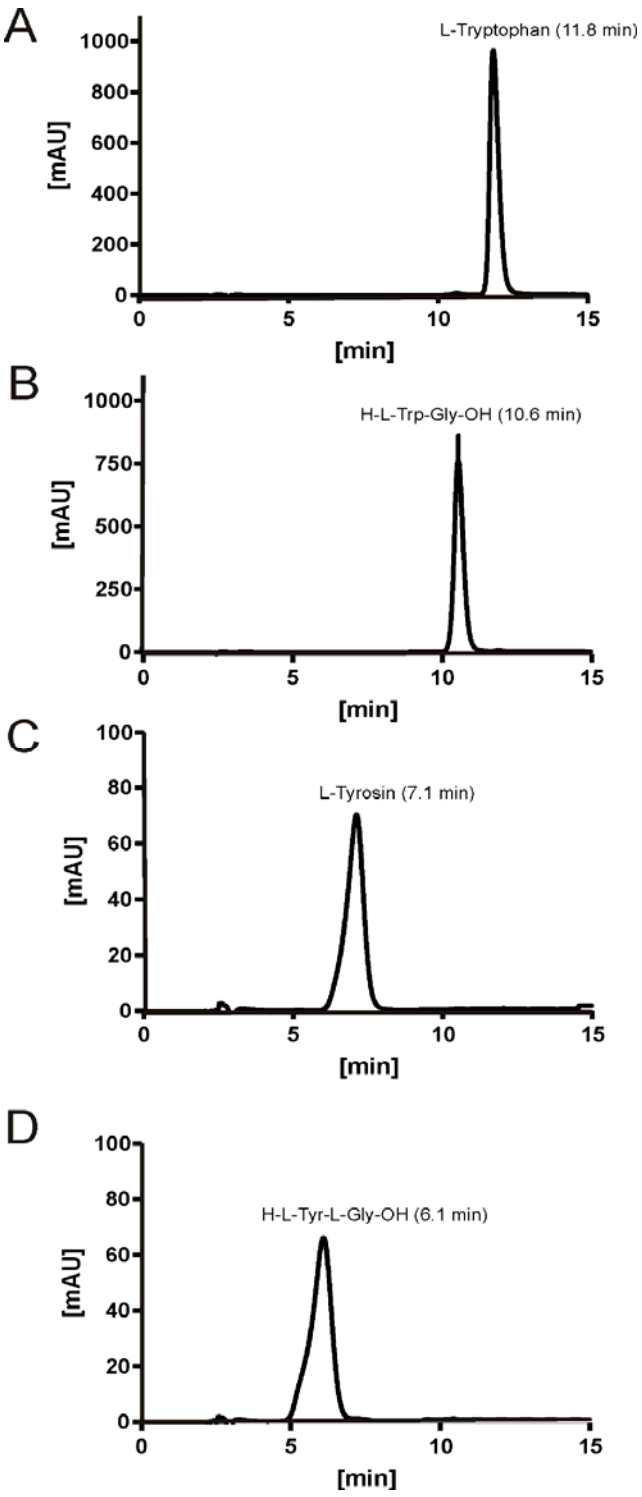

Abb. 1.7: HPLC-Chromatogramme der Versuche zur Hydrolyseaktivität von SirD. A: Inkubation von SirD mit H-L-Trp-Gly-OH; B: Inkubation mit hitze-denaturiertem SirD mit H-L-Trp-Gly-OH; C: Inkubation von SirD mit H-L-Tyr-Gly-OH; D: Inkubation mit hitze-denaturiertem SirD mit H-L-Tyr-Gly-OH. Die Assays enthielten alle 48 nM SirD und 2 mM des jeweiligen Substrates und wurden für 16 Stunden bei 37°C inkubiert.

Für eine Zusammenfassung der Ergebnisse wird auf folgende Publikation verwiesen:

Kremer, A. and Li, S.-M. (2009)

A tyrosine O-prenyltransferase catalyses the first pathway-specific step in the biosynthesis of sirodesmin PL

Microbiology, im Druck

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4 Anhang

4.1 Veröffentlichungen

- Kremer, A., Westrich, L. and Li, S.-M. (2007)
  A 7-dimethylallyltryptophan synthase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterization
  Microbiology 153, 3409-3416

- Kremer, A. and Li, S.-M. (2008)
  Potential of a 7-dimethylallyltryptophan synthase as a tool for production of prenylated indole derivatives
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- Kremer, A. and Li, S.-M. (2008)
  Tryptophan aminopeptidase activity of several indole prenyltransferases from *Aspergillus fumigatus*
  Chem. Biol. 15, 729-738

- Kremer, A. and Li, S.-M. (2009)
  A tyrosine O-prenyltransferase catalyses the first pathway-specific step in the biosynthesis of sirodesmin PL
  Microbiology, im Druck

- Kremer, A., Stec, E., Yin, W.-B., Grundmann, A. and Li, S.-M. (2009)
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A 7-dimethylallyltryptophan synthase from *Aspergillus fumigatus*: overproduction, purification and biochemical characterization

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A putative prenyltransferase gene, *Afu3g12930*, was identified in the genome sequence of *Aspergillus fumigatus*. EAL92290, encoded by *Afu3g12930*, consists of 472 aa, with a molecular mass of about 53 kDa. The coding sequence of *Afu3g12930* was cloned in pQE60, and overexpressed in *Escherichia coli*. The soluble His_6-fusion protein was purified to apparent homogeneity, and characterized biochemically. The enzyme was found to catalyse the prenylation of Trp at the C-7 position of the indole moiety, in the presence of dimethylallyl diphosphate (DMAPP); therefore, it functions as a 7-dimethylallyltryptophan synthase (7-DMATS). The structure of the enzymic product was elucidated by NMR and MS analysis. K_m values were 67 μM for DMAPP, and 137 μM for L-Trp. Geranyl diphosphate was not accepted as prenyl donor, while Trp-containing dipeptides were found to be aromatic substrates of 7-DMATS. 7-DMATS did not need divalent metal ions for its enzymic reaction, although Ca^{2+} enhanced the reaction velocity slightly. The enzyme is the second dimethylallyltryptophan synthase identified in *A. fumigatus*. Interestingly, it shares a sequence identity of only 31 % at the amino acid level with another known dimethylallyltryptophan synthase, FgaPT2, from the same fungus; FgaPT2 prenylates L-Trp at the C-4 position of the indole ring. *Afu3g12930* belongs to a putative biosynthetic gene cluster consisting of eight genes. Orthologous clusters were also identified in the genome sequences of *Neosartorya fischeri* and *Aspergillus terreus*. The putative roles of the genes in the cluster are discussed.

INTRODUCTION

Like bacteria and plants, fungi produce pharmacologically important agents (Keller *et al.*, 2005), for example: the immunosuppressive cyclosporin A is obtained from *Tolypocladium inflatum* (Thali, 1995); statins are produced by several fungi, including *Aspergillus terreus* (Manzoni & Rollini, 2002); penicillins are produced by *Penicillium chrysogenum* and *Aspergillus nidulans* (Brakhage *et al.*, 2004); and ergot alkaloids from *Claviceps purpurea* show diverse pharmacological activities (Schardl *et al.*, 2006). Secondary metabolites from fungi are usually active as mycotoxins and virulence factors (Bennett & Klich, 2003; Rementeria *et al.*, 2005) that could be involved in the pathogenic development of fungal infections. *Aspergillus fumigatus*, a filamentous fungal saprophyte found ubiquitously in the environment, is responsible for allergenic reactions and invasive aspergillosis, which is a life-threatening disease for immunocompromised patients (Denning *et al.*, 2003). It has been discussed that the virulence of *A. fumigatus* could be based on its numerous secondary metabolites, especially gliotoxin (Latgé, 1999), which is an epipolythiodioxopiperazine (ETP) derived from Phe and Ser (Gardiner & Howlett, 2005). However, the role of these compounds remains questionable, and needs to be investigated more thoroughly (Latgé, 1999). ETPs are characterized by the presence of an internal disulphide bridge (Gardiner *et al.*, 2005). The role of gliotoxin in the pathogenic development of *A. fumigatus* has been demonstrated by deleting the non-ribosomal peptide synthetase (NRPS) gene *gliP* from the gliotoxin biosynthetic gene cluster (Cramer *et al.*, 2006a; Kupfahl *et al.*, 2006), and it was clearly demonstrated that gliotoxin production was blocked in the resulting mutants; however, the abolition of gliotoxin production had no effect on the development of invasive aspergillosis by the mutants

Abbreviations: DMAPP, dimethylallyl diphosphate; DMAT, dimethylallyltryptophan; DMATS, dimethylallyltryptophan synthase; ETP, epipolythiodioxopiperazine; GPP, geranyl diphosphate; NRPS, non-ribosomal peptide synthetase.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence reported in this paper is EF539173.

(Cramer *et al.*, 2006a; Kupfahl *et al.*, 2006). This proved that gliotoxin is not, or at least not alone, responsible for the pathogenic development in *A. fumigatus*. Therefore, it would be useful to identify genetic information of further secondary metabolites, which could function as virulence factors.

From the genome sequence of *A. fumigatus* Af293, at least 26 biosynthetic gene clusters for secondary metabolites have been identified (Nierman *et al.*, 2005), including the gene clusters of fumitremorgin B and fumigaclavine C, as well as that of gliotoxin (Cramer *et al.*, 2006b; Gardiner & Howlett, 2005; Grundmann & Li, 2005; Maiya *et al.*, 2006; Unsöld & Li, 2005). One putative gene cluster has been proposed for the biosynthesis of an additional unknown ETP derivative (Cramer *et al.*, 2006b). This putative gene cluster contains three genes showing significant sequence similarity to *gliC, gliP* and *gliM* of the gliotoxin cluster of *A. fumigatus* (Gardiner & Howlett, 2005). Homologues of these genes, i.e. *sirC, sirP* and *sirM*, have also been found in the gene cluster of sirodesmin, which is an ETP derivative from the phytopathogenic fungus *Leptosphaeria maculans* (Gardiner *et al.*, 2004). The gene cluster of sirodesmin contains a putative prenyltransferase gene, *sirD*, which has been proposed to be responsible for the *O*-prenylation of L-Tyr or *cyclo*-L-Tyr-L-Ser (Gardiner *et al.*, 2004, 2005). A putative prenyltransferase gene, *Afu3g12930*, has also been found in the cluster from *A. fumigatus*, and the protein that it encodes shares a sequence identity of 34 % with SirD at the amino acid level; this is higher than the sequence similarity to other fungal prenyltransferases, or other entries in the databases, with the exception of its orthologues in *Neosartorya fischeri* and *A. terreus* (see Discussion). Based on this sequence similarity, it could be speculated that EAL92290, encoded by *Afu3g12930*, would catalyse the transfer reaction of a prenyl moiety to Tyr or its derivatives. To prove the function of *Afu3g12930*, we cloned and overexpressed this gene in *Escherichia coli*, and carried out biochemical investigation using the purified fusion protein. Our results showed, interestingly, that *Afu3g12930* encodes a second dimethylallyltryptophan synthase (DMATS), termed 7-DMATS, in *A. fumigatus*, and this enzyme catalyses the prenylation of Trp instead of Tyr. The first DMATS (FgaPT2) identified in *A. fumigatus* is involved in the biosynthesis of fumigaclavine C; in contrast to this, 7-DMATS catalyses the prenylation of Trp at the C-7 position, instead of at the C-4 position, of the indole ring.

METHODS

Computer-assisted sequence analysis. FGENESH (Softberry; www.softberry.com/berry.phtml) and the DNASIS software package (version 2.1; Hitachi Software Engineering) were used for intron prediction and sequence analysis, respectively. Sequence similarities were obtained by alignments of amino acid sequences using the BLAST 2 sequences program (release 2.9.9; http://www.ncbi.nlm.nih.gov/blast/bl2seq/wblast2.cgi).

Chemicals. The trisammonium salt of dimethylallyl diphosphate (DMAPP) was synthesized using methods analogous to those used for the synthesis of trisammonium geranyl diphosphate reported by Woodside *et al.* (1988). Geranyl diphosphate (GPP) was kindly provided by Wessjohann. The peptides used in this study were purchased from Bachem.

Bacterial strains, plasmids and culture conditions. pGEM-T and pQE60 were obtained from Promega and Qiagen, respectively. A Uni-ZAP XR premade library of *A. fumigatus* strain B5233 (ATCC 13073) was purchased from Stratagene, and used to obtain phagemids as cDNA templates for PCR amplification. *E. coli* XL1 Blue MRF′ (Stratagene) was used for cloning and expression experiments, and it was grown in liquid Luria–Bertani (LB) medium, or on solid LB medium with 1.5 % (w/v) agar, at 37 °C (Sambrook & Russell, 2001). Addition of 50 μg carbenicillin ml^{-1} was used for selection of recombinant *E. coli* strains.

DNA isolation, PCR amplification and cloning. Standard procedures for DNA isolation and manipulation were performed, as described (Sambrook & Russell, 2001). PCR amplification was carried out on an iCycler from Bio-Rad. Using the Expand High Fidelity kit (Roche Diagnostics), a PCR fragment of 1425 bp, containing the entire coding sequence of *Afu3g12930*, was amplified from the cDNA library by using the primers 7-dmats-1 (5′-CA**C**CATG**G**CCA-TCGGAGCCGAGAT-3′) at the 5′ end, and 7-dmats-2 (5′-TGC**AGATCT**GCTGTACACCCGGAG-3′) at the 3′ end of the gene. Bold letters represent mutations inserted to give the underlined restriction sites *Nco*I, located at the start codon in 7-dmats-1, and *Bgl*II, located at the predicted stop codon in 7-dmats-2. The PCR fragment was cloned into pGEM-T, resulting in plasmid pLW39, which has subsequently had its sequence confirmed (MWG-Biotech). To create the expression vector pLW40, pLW39 was digested with *Nco*I and *Bgl*II, and the resulting *Nco*I–*Bgl*II fragment of 1418 bp was ligated into pQE60, which had been digested with the same enzymes.

Overproduction and purification of His_6-7-DMATS protein. For gene expression, *E. coli* XL1 Blue MRF′ cells harbouring the plasmid pLW40 were cultivated in 300 ml Erlenmeyer flasks containing 100 ml liquid LB medium, supplemented with carbenicillin (50 μg ml^{-1}), and grown at 37 °C to an OD_{600} of 0.6. For induction, IPTG was added to a final concentration of 0.8 mM, and the cells were cultivated for a further 16 h at 37 °C, before harvest. The bacterial cultures were centrifuged, and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0) at between 2 and 5 ml (g wet weight)$^{-1}$. After addition of 1 mg lysozyme ml^{-1}, and incubation on ice for 30 min, the cells were sonicated six times, for 10 s each time, at 200 W. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 14 000 ***g*** for 30 min at 4 °C. One-step purification of the recombinant His_6-tag fusion protein by affinity chromatography with Ni-NTA agarose resin (Qiagen) was carried out according to the manufacturer's instructions. The protein was eluted with 250 mM imidazole in 50 mM NaH_2PO_4 and 300 mM NaCl, pH 8.0. In order to change the buffer, the protein fraction was passed through a NAP-5 column (GE Healthcare), which had been equilibrated with 50 mM Tris/HCl and 15 % (v/v) glycerol, pH 7.5. His_6-7-DMATS was eluted with the buffer described, and stored at −80 °C for enzyme assays.

Protein analysis. Proteins were analysed by SDS-PAGE, which was carried out according to the method of Laemmli (1970), and they were stained with Coomassie brilliant blue G-250.

Assays for 7-DMATS activity. All the enzyme assays contained 50 mM Tris/HCl, pH 7.5, 1.2–3.8 % (v/v) glycerol, and 10 mM $CaCl_2$. They differed from each other by incubation volume and time, substrate concentration, and amount of recombinant 7-DMATS. The reaction mixtures were incubated at 37 °C, and the reactions were

terminated by addition of 10 µl TCA (1.5 M) per 100 µl reaction volume. After removal of the protein by centrifugation (14 000 ***g***, 10 min, 4 °C), the enzymic products were analysed on an HPLC system, as described below. The standard assays for determination of the substrate specificity (100 µl) contained 2 mM L-Trp or another aromatic substrate, 1 mM DMAPP and 2.5 µg purified 7-DMATS, and they were incubated for 45 min. Two independent assays were carried out for quantification. The assay for the isolation of the enzymic product for structural elucidation (5 ml) contained 400 µg purified 7-DMATS, and was incubated for 16 h. The reaction mixture was concentrated on a rotation evaporator at 35 °C to a volume of 750 µl, before injection of 100 µl into HPLC for isolation of 7-dimethylallyltryptophan (7-DMAT). The assays for determination of the kinetic parameters of L-Trp contained 1 mM DMAPP, 2.5 µg 7-DMATS, and L-Trp at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5, 1.0, 2.0, 3.0 or 5.0 mM, in a total volume of 100 µl. The incubation time was 45 min. For determination of the kinetic parameters of DMAPP, 2.5 µg 7-DMATS, 1 mM L-Trp, and DMAPP at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5 or 1.0 mM, were used.

HPLC analysis and isolation of 7-DMAT. Reaction mixtures of 7-DMATS were analysed on an Agilent HPLC Series 1100 by using an Eclipse XBD-C18 column (4.6 × 150 mm, 5 µm) at a flow rate of 1 ml min^{-1}. Water (solvent A) and acetonitrile (solvent B), each containing 0.5 % (v/v) TFA, were used as solvents. A gradient was run from 15 to 70 % solvent B in 15 min. After washing with 100 % solvent B for 5 min, the column was equilibrated with 85 % solvent A for 5 min. The substances were detected with a Photo Diode Array detector. 7-DMAT was isolated under the same conditions.

Spectroscopic data. A ^{1}H-NMR spectrum of 7-DMAT was taken on an Avance DRX 500 spectrometer (Bruker) using 0.1 M DCl in D_2O as the solvent. The solvent signal at 4.81 p.p.m. was used as reference. δ (p.p.m.): 7.53 (d, 7.6 Hz, H-4), 7.32 (s, H-2), 7.12 (t, 7.9 Hz, H-5), 7.10 (d, 8.2, H-6), 5.46 (br t, 7.0 Hz, H-2′), 4.39 (t, 6.1, H-11), 3.58 (d, 7.0 Hz, 2H-1′), 3.52 (dd, 15.5, 5.4 Hz, H-10), 3.43 (dd, 15.5, 7.0 Hz, H-10), 1.74 (s, 3H-5′), 1.72 (s, 3H-4′).

Positive and negative electrospray ionization (ESI) mass spectra were obtained with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled with an Agilent HPLC series 1100 equipped with an RP18-column (2 × 250 mm, 5 µm). For separation, the column was run with 10 % solvent B (CH_3OH) in solvent A (H_2O) [each containing 0.1 % (v/v) HCOOH] for 5 min, followed by a gradient from 10 to 100 % solvent B over 30 min. After washing with 100 % solvent B for 10 min, the column was equilibrated with 10 % solvent B for 10 min. The flow rate was at 0.2 ml min^{-1}.

MS data of 7-DMAT. Positive ESI-MS: $[M+H]^+$: *m/z* 273.1; ms^2 of $[M+H]^+$; *m/z* (%): 272.9 (5), 256.0 (100), 240.8 (9); 214.0 (64), 212.0 (24), 199.9 (25), 156.0 (6). Negative ESI-MS: $[M-H]^-$: *m/z* 271.2; ms^2 of $[M-H]^-$; *m/z* (%): 271.2 (100), 227.3 (40), 210.3 (65); 184.3 (94).

MS data of the prenylated H-L-Trp-L-Gly-OH. Positive ESI-MS: $[M+H]^+$: *m/z* 330.2; ms^2 of $[M+H]^+$; *m/z* (%): 313.0 (25), 271.1 (34), 257.0 (41), 244.9 (16), 238.0 (47), 227.0 (7), 212.0 (100), 198.1 (25), 156.1 (11). Negative ESI-MS: $[M-H]^-$: *m/z* 328.4; ms^2 of $[M-H]^-$; *m/z* (%): 328.2 (100), 284.3 (31), 225.3 (6), 198.2 (10), 131.1 (39).

MS data of the prenylated *cyclo*-L-Trp-L-Gly. Positive ESI-MS: $[M+H]^+$: *m/z* 312.2; ms^2 of $[M+H]^+$; *m/z* (%): 256.0 (9), 198.1 (100). Negative ESI-MS: $[M-H]^-$: *m/z* 310.6; ms^2 of $[M-H]^-$; *m/z* (%): 310.2 (31), 113.1 (100).

RESULTS

Sequence analysis of the putative prenyltransferase gene *Afu3g12930* from *A. fumigatus*

The nucleotide sequence of the genomic DNA from *A. fumigatus* Af293 is available at GenBank under accession number AAHF01000002.1, and the genomic sequence of the putative prenyltransferase gene *Afu3g12930* spans bp 640 080–641 551, and consists of two exons of 1301 and 118 bp, respectively, interrupted by an intron of 53 bp; this was confirmed by sequencing the PCR products amplified from the cDNA of *A. fumigatus* strain B5233 (data not shown; GenBank accession no. EF539173). The predicted gene product of *Afu3g12930* is EAL92290, which comprises 472 aa, and has a calculated molecular mass of 53 kDa. EAL92290 (7-DMATS) shows significant sequence similarity to aromatic prenyltransferases from various fungi. For example, by using the BLAST 2 sequences program, we found that 7-DMATS shares a sequence identity of 34 % with SirD from *L. maculans* (Gardiner & Howlett, 2005) at the amino acid level, 31 % with FgaPT2 (DMATS) from *A. fumigatus* (Unsöld & Li, 2005), 28 % with DMATS from *C. purpurea* and *Claviceps fusiformis* (Tsai *et al.*, 1995; Tudzynski *et al.*, 1999), and 28 % with FtmPT1 (Grundmann & Li, 2005) and 26 % with FgaPT1 (Unsöld & Li, 2006), which are both from *A. fumigatus*. SirD is proposed to catalyse the *O*-prenylation of L-Tyr or *cyclo*-L-Tyr-L-Ser in the biosynthesis of sirodesmin (Gardiner *et al.*, 2004). DMATS from different sources catalyse the prenylation of L-Trp at the C-4 position of the indole ring in the biosynthesis of ergot alkaloids (Li & Unsöld, 2006; Schardl *et al.*, 2006). FtmPT1 and FgaPT1 have been found to catalyse the prenylation reactions at the C-2 position of the indole rings of brevianamide F and fumigaclavine A (Grundmann & Li, 2005; Unsöld & Li, 2006), respectively. Based on the high sequence similarity to SirD, we speculate that 7-DMATS could be responsible for a transfer reaction of a prenyl moiety to L-Tyr or its derivatives.

Cloning of *Afu3g12930*, and overproduction and purification of His_6-7-DMATS

The coding region of *Afu3g12930* was amplified using PCR from cDNA of *A. fumigatus* strain B5233 (available in the form of phagemids isolated from a cDNA library), and cloned into the cloning vector pGEM-T. For gene expression, the coding sequence of *Afu3g12930* was released from pGEM-T, and cloned into the vector pQE60 (see Methods), resulting in the expression plasmid pLW40.

E. coli cells harbouring pLW40 were induced by 0.8 mM IPTG at 37 °C. His_6-7-DMATS was purified with Ni-NTA agarose to apparent homogeneity, as judged by SDS-PAGE (Fig. 1), and a protein yield of 5 mg purified His_6-tagged 7-DMATS per litre of culture was obtained. The observed molecular mass was 50 kDa, and this corresponded well to the calculated value of 54 kDa for His_6-7-DMATS.

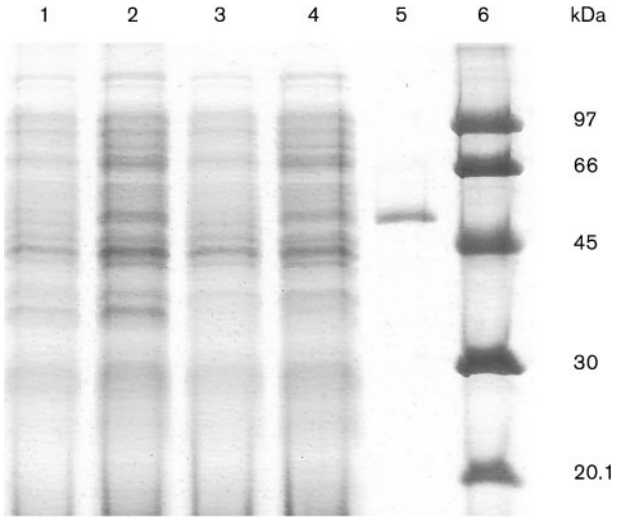

Fig. 1. Analysis of the overproduction and purification of His_6-7-DMATS. The proteins were separated on a 12% SDS-polyacrylamide gel, and stained with Coomassie brilliant blue G-250. Lanes: 1, total proteins before induction; 2, total proteins after induction with 0.8 mM IPTG at 37 °C for 16 h; 3, soluble proteins before induction; 4, soluble proteins after induction; 5, purified His_6-7-DMATS; 6, molecular mass standard.

Enzymic activity and substrate specificity of 7-DMATS

Initial attempts to detect the enzymic activity were carried out by incubations of 7-DMATS with L-Tyr and *cyclo*-L-Tyr-L-Ser in the presence of DMAPP or GPP. HPLC analysis was used to monitor the formation of an enzymic product. However, no product peak could be detected under different conditions, including various substrate concentrations and amounts of purified 7-DMATS, different metal ions with various concentrations, and different pH values and buffer compositions (data not shown). After the unsuccessful attempts, we decided to test other aromatic amino acids and their derivatives. HPLC analysis of the incubation mixture of L-Trp and DMAPP showed a significant product peak with a retention time of 12.9 min; this peak was absent in the reaction mixture containing heat-denaturated enzyme (Fig. 2). Dependence of the product formation on the amount of protein was found up to 4 μg per 100 μl assay, and on a reaction time of up to 50 min. Product formation was strictly dependent on the presence of His_6-7-DMATS, L-Trp and DMAPP. The enzyme 7-DMATS was found to be specific for DMAPP. Product formation was observed with DMAPP only, and not with GPP. In contrast, 7-DMATS showed relative flexible substrate specificity towards its aromatic substrates. D-Trp was accepted by 7-DMATS, with a relative activity of 15.5% of its enantiomer L-Trp. Linear and cyclic dipeptides were also substrates of 7-DMATS. Product formation was clearly observed when H-L-Trp-L-Gly-OH or *cyclo*-L-Trp-L-Gly was used instead of L-Trp in the incubation mixtures (Table 1). However, the linear tripeptide L-Trp-L-Trp-L-Trp was not accepted by 7-DMATS. No product formation was observed in incubation mixtures with L-Phe, or L-Phe-containing cyclic dipeptides (Table 1). These results demonstrated that

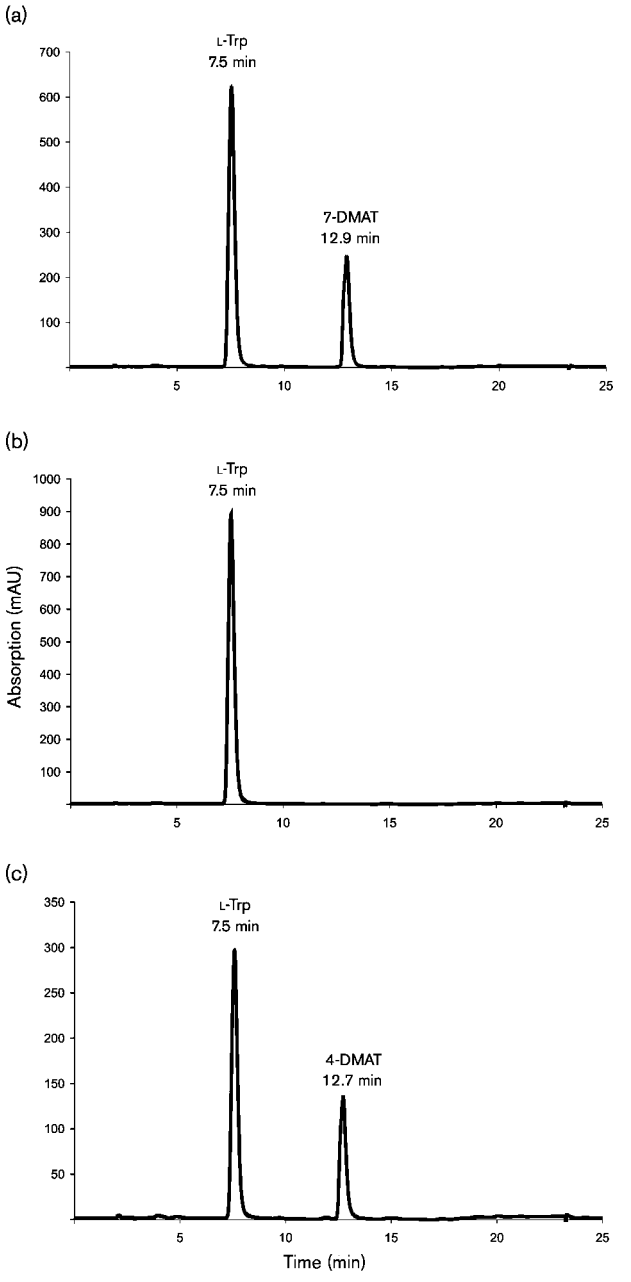

Fig. 2. HPLC chromatograms of enzyme assays with His_6-7-DMATS (a), with heat-inactivated His_6-7-DMATS by boiling of the enzyme solution for 20 min (b), and with His_6-FgaPT2 (c). The reaction mixtures contained 1 mM DMAPP, L-Trp (2 mM for 7-DMATS assays, and 1 mM for the FgaPT2 assay), and 4 μg recombinant 7-DMATS or 1 μg recombinant FgaPT2, and they were incubated for 16 h. The substances were detected with a Photo Diode Array detector, and illustrated at 277 nm.

Table 1. Substrate specificity of 7-DMATS towards different aromatic substrates

For incubation conditions, see Methods.

Substrate	Relative activity (%)
L-Trp	100.0 ± 1.5
D-Trp	15.5 ± 3.1
H-L-Trp-L-Gly-OH	10.9 ± 1.1
cyclo-L-Trp-L-Gly	1.8 ± 0.2
H-L-Trp-L-Trp-L-Trp-OH	<0.3
L-Tyr	<0.3
cyclo-L-Tyr-L-Pro	<0.3
cyclo-L-Tyr-L-Ser	<0.3
L-Phe	<0.3
cyclo-L-Phe-L-Phe	<0.3
cyclo-L-Phe-L-His	<0.3

L-Trp was the best substrate for 7-DMATS, under our test conditions.

Identification of the enzymic products

For structural elucidation, the enzymic product of L-Trp was isolated on a preparative scale, and its structure was elucidated by NMR and MS analysis.

ESI-MS proved that the enzymic product is a prenylated derivative of Trp by detection of $[M+H]^+$ and $[M-H]^-$ at *m/z* 273 and *m/z* 271, respectively. Comparison of the ^{1}H-NMR spectrum of the isolated compound with that of L-Trp revealed the presence of signals for a dimethylallyl moiety at 5.46 (br t, 7.0 Hz, H-2′), 3.58 (d, 7.0 Hz, 2H-1′), 1.74 (s, 3H-5′) and 1.72 p.p.m. (s, 3H-4′). Correspondingly, signals for four protons instead of five protons were found in the region of the aromatic protons of the isolated compound, i.e. signals for three vicinal protons at 7.53 (d, 7.6 Hz), 7.12 (t, 7.9 Hz) and 7.10 p.p.m. (d, 8.2), and a singlet at 7.32 p.p.m. This indicated that the prenylation had taken place at the C-4 or C-7 position of the indole moiety. The spectrum of the isolated compound differed clearly from that of 4-DMAT for all of the protons, with the exception of the proton at C-2 (Unsöld & Li, 2005). 4-DMAT, the first pathway-specific intermediate in the biosynthesis of ergot alkaloids (Schardl *et al.*, 2006; Unsöld & Li, 2005), is also formed from L-Trp by a prenyl-transfer reaction catalysed by DMATS, e.g. FgaPT2 from *A. fumigatus* (Unsöld & Li, 2005). The enzymic product of 7-DMATS showed a similar, but different, retention time to that of FgaPT2 on the HPLC chromatogram (Fig. 2). Therefore, the enzymic product of 7-DMATS was identified as 7-DMAT (Fig. 3). The enzymic products of H-L-Gly-L-Trp-OH and *cyclo*-L-Trp-L-Gly were unequivocally proven as prenylated derivatives by detection of $[M+H]^+$ at *m/z* 330.2 and 312.2, as well as $(M-H)^-$ at *m/z* 328.4 and 310.6, respectively.

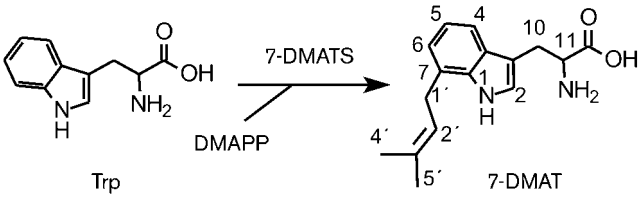

Fig. 3. Enzymic reaction catalysed by 7-DMATS.

Biochemical properties and kinetic parameters of 7-DMATS

Testing with different ions showed that metal ions, such as Mg^{2+} or Mn^{2+}, are not essential for the prenyltransferase activity of 7-DMATS. In the presence of 10 mM EDTA, a chelating agent for divalent ions, a relative activity of 82 % was observed, in comparison with the incubation mixture without additives. However, an increase in enzyme activity of up to 140 % of that of the incubation without additives was detected in the presence of 10 mM Ca^{2+}. This finding is similar to those of some of the soluble aromatic prenyltransferases, e.g. CloQ from *Streptomyces roseochromogenes* (Pojer *et al.*, 2003), LtxC from *Lyngbya majuscula* (Edwards & Gerwick, 2004), DMATS from *C. purpurea* (Gebler & Poulter, 1992), and FgaPT1, FgaPT2 and FtmPT1 from *A. fumigatus* (Grundmann & Li, 2005; Unsöld & Li, 2005, 2006).

The 7-DMATS reaction apparently followed Michaelis–Menten kinetics. By using Hanes–Woolf and Lineweaver–Burk plots, the K_m values were determined as 137 μM for L-Trp, and 67 μM for DMAPP. The maximum reaction velocity observed with 7-DMATS was 0.21 μmol min^{-1} mg^{-1}. These values are in the normal range of those of enzymes involved in the biosynthesis of secondary metabolites. Given that L-Trp was shown to be the best substrate in our test conditions (see above), it is very likely to be the natural substrate of 7-DMATS.

DISCUSSION

In this study, we cloned and overexpressed the putative prenyltransferase gene *Afu3g12930* from the fungus *A. fumigatus* in a heterologous host, and proved its function by biochemical characterization of the overproduced enzyme. Investigation of substrate specificity showed that 39 of 53 compounds were accepted by 7-DMATS, with the highest conversion rate for L-Trp. K_m values of six Trp derivatives, which had modifications to the side chain and the indole ring, were determined to be in the range of 190–460 μM, which is higher than that for L-Trp (137 μM). K_m values of eight linear and cyclic Trp-containing dipeptides were found to be from 180 to 500 μM (Kremer & Li, unpublished data). This indicated that the natural substrate of 7-DMATS is very likely to be L-Trp. However, it cannot be excluded that an unidentified free or enzyme-bound

dipeptide containing a Trp moiety (see below for the discussion on the role of the NRPS) is the natural substrate of the identified enzyme. The enzymic product of 7-DMATS was unequivocally identified as 7-DMAT by NMR and MS analysis. This demonstrated clearly that 7-DMATS functions as a dimethylallyltryptophan synthase. Interestingly, 7-DMATS shares a sequence identity of only 31 % at the amino acid level with another DMATS, FgaPT2 (Unsöld & Li, 2005), from the same fungus; this level of identity is slightly higher than the sequence similarities between 7-DMATS and other indole prenyltransferases from *A. fumigatus*, e.g. 7-DMATS shares a sequence identity of 28 % with FtmPT1, which catalyses the prenylation of the cyclic dipeptide *cyclo*-L-Trp-L-Pro at the C-2 position of the indole moiety (Grundmann & Li, 2005), and 26 % with FgaPT1, which prenylates fumigaclavine A at the C-2 position of the indole moiety (Unsöld & Li, 2006). 7-DMATS accepted Trp, but not Tyr as a substrate. However, with the exception of its orthologues in *N. fischeri* NRRL181 and *A. terreus* NIH2624, with sequence identities of 95 and 82 % (Table 2, see below) at the amino acid level, respectively, 7-DMATS showed the highest sequence similarity to SirD from the biosynthetic gene cluster of sirodesmin from *L. maculans* (Gardiner *et al.*, 2004), i.e. a sequence identity of 34 % at the amino acid level. SirD has been proposed to catalyse the *O*-prenylation of L-Tyr, or its cyclic dipeptide, with Ser (Gardiner *et al.*, 2005). It would be interesting to prove the function of this putative prenyltransferase biochemically.

Divalent metal ions, such as Mg^{2+} and Mn^{2+}, are not essential for the enzymic reaction of 7-DMATS. However, like other indole prenyltransferases, such as FgaPT1, FgaPT2 and FtmPT1 mentioned above (Grundmann & Li, 2005; Unsöld & Li, 2005, 2006), as well as DMATS in the biosynthesis of ergot alkaloids from *Claviceps* (Lee *et al.*, 1976), Ca^{2+} enhanced the enzymic reaction of 7-DMATS slightly. This finding, together with the sequence similarity, provided experimental data for the hypothesis that the indole prenyltransferases from fungi belong to the same group of enzymes, and that they have evolved directly from a common ancestor.

Afu3g12930 belongs to a putative biosynthetic gene cluster consisting of probably eight genes (Fig. 4, Table 2): two putative transcription factor genes *Afu3g12940* and *Afu3g12890*, one putative transporter gene (*Afu3g12900*), one gene with unknown function (*Afu3g12950*), and four biosynthetic genes, i.e. the NRPS gene *Afu3g12920*, the prenyltransferase gene *Afu3g12930* described in this study, a gene coding for a putative cytochrome P450 enzyme (*Afu3g12960*), and a putative methyltransferase gene *Afu3g12910*. With the exception of *Afu3g12950*, orthologous genes were also found in the genome sequence of *N. fischeri* NRRL181 and *A. terreus* NIH2624, with the same orientation and same relative position of the genes (GenBank entries) (Fig. 4). The gene products of the cluster from *A. fumigatus* share sequence identities of 92–96 % at the amino acid level with their orthologues from *N. fischeri*, and 73–88 % with the orthologues from *A. terreus* (Table 2), respectively. The gene *Afu3g12950* in the gene cluster from *A. fumigatus* encodes a putative ubiquitin-conjugating enzyme. An orthologue of this gene was found in the gene cluster of *N. fischeri*, but not in the gene cluster of *A. terreus*. Therefore, it is questioned whether *Afu3g12950* is essential for the biosynthesis of the compound encoded by this gene cluster.

The end product of the eight-gene cluster is unknown (Cramer *et al.*, 2006b; Nierman *et al.*, 2005). Deletion of the NRPS gene *Afu3g12920* from the genome of *A. fumigatus* CEA17Δ*akuB* (Silva Ferreira *et al.*, 2006) by T. Heinekamp and A. A. Brakhage (HKI, Jena, Germany), and HPLC analysis of the cultural extracts by our group, showed that no changes of the profile of the secondary metabolite production could be observed in the obtained mutants, in comparison with that of the wild-type (data not shown). This indicated that one or more genes of this cluster were not expressed, or were expressed at very low levels, so that no product of this cluster accumulated in the

Table 2. ORFs of the putative cluster in *A. fumigatus* Af293

A. fumigatus ORF	Identity* to genes of				Putative function
	N. fischeri	*A. terreus*	Gliotoxin cluster	Sirodesmin cluster	
EAL92287	EAW21272 (92)	EAU31603 (85)	GliC (32)	SirC (39)	Cytochrome P450 monooxygenase
EAL92288	EAW21273 (95)	–	–	–	Ubiquitin conjugating enzyme
EAL92289	EAW21274 (96)	EAU31602 (84)	–	–	C-6 transcription factor
EAL92290 (7-DMATS)	EAW21275 (95)	EAU31601 (82)	–	SirD (34)	Prenyltransferase
EAL92291	EAW21276 (93)	EAU31600 (76)	GliP (29)	SirP (36)	NRPS containing A1-T1-C1-A2-T2-C2-T3
EAL92292	EAW21277 (96)	EAU31599 (88)	GliM (34)	SirM (38)	Methyltransferase
EAL92293	EAW21278 (96)	EAU31598 (87)	–	–	Transporter
EAL92294	EAW21279 (93)	EAU31597 (73)	GliZ (27)	SirZ (23)	C-6 transcription factor

*Percentage similarity values of the *A. fumigatus* ORFs to those of *N. fischeri*, *A. terreus*, and the gliotoxin and sirodesmin clusters, are given in parentheses.

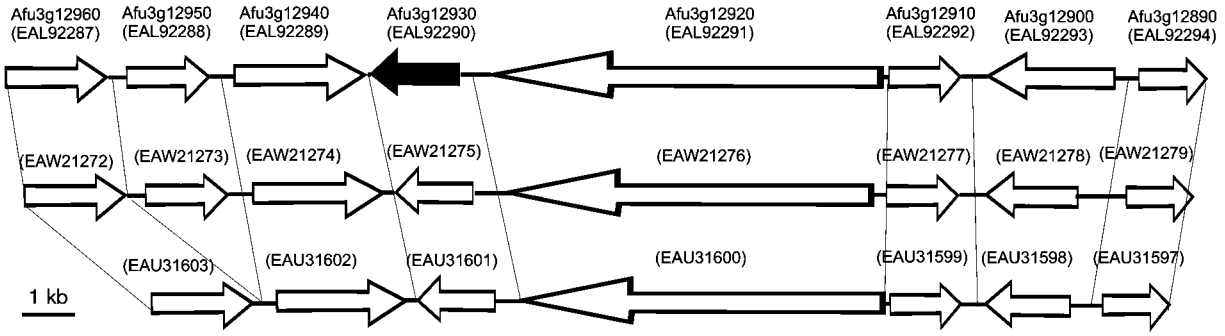

Fig. 4. Putative unknown gene cluster from different *Aspergillus* strains. Top, *A. fumigatus* Af293; middle, *N. fischeri* (*Aspergillus fischerianus*) NRRL181; bottom, *A. terreus* NIH2624. Introns are not shown. The deduced protein names are given in parentheses.

wild-type, and, therefore, no effect on secondary metabolite production could be observed after deletion of a gene from the cluster. This finding is in accordance with the results reported by Cramer *et al.* (2006b) that *Afu3g12920* is not expressed, or is expressed at very low levels, under the conditions tested in their study. However, it can not be excluded that this cluster could be expressed *in situ* after inhalation of the fungus, and the secondary metabolite may then play a role as virulence factor.

Comparison with database entries revealed that the four biosynthesis enzymes EAL92287, EAL92290 (7-DMATS), EAL92291 and EAL92292 share sequence identities of 39, 34, 36 and 38 % with SirC, SirD, SirP and SirM, respectively (Table 2), which have been identified in the gene cluster of sirodesmin, which is an ETP from *L. maculans* (Gardiner *et al.*, 2004). Sequence identities of 32, 29 and 34 % were also observed between EAL92287 and GliC, EAL92291 and GliP, as well as EAL92293 and GliM, respectively (Table 2). GliP, GliC and GliM are members of the gene cluster of the ETP gliotoxin from *A. fumigatus* (Gardiner & Howlett, 2005). The sequence identities of EAL92294 to SirZ and GliZ were found to be 23 and 27 %, respectively (Table 2). Therefore, the cluster discussed here is proposed to function in the biosynthesis of an ETP derivative (Cramer *et al.*, 2006b; Nierman *et al.*, 2005).

However, database and literature searches revealed that none of the known ETP derivatives contain a prenylated Trp moiety (Gardiner *et al.*, 2005). Also, and more importantly, a homologue of the putative thioredoxin reductase, GliT/SirT, in the gene cluster of gliotoxin/sirodesmin, which is proposed to be responsible for the formation of the disulphide bond of the ETP derivatives (Gardiner *et al.*, 2005; Gardiner & Howlett, 2005), is absent in the cluster. Therefore, it could be speculated that the cluster discussed here is not intact, or that the product of the gene cluster has a structure other than an ETP or a derivative thereof. Considering the fact that the cluster is present in the three fungi mentioned above, it seems that the latter is the most plausible explanation. The product of the gene cluster could be a derivative of a cyclic dipeptide containing 7-DMAT. It is plausible that L-Trp is converted to 7-DMAT, and that this is catalysed by the prenyltransferase 7-DMATS characterized in this study. 7-DMAT would then undergo the condensation with a second amino acid, which could not be predicted by the sequence analysis, to a prenylated (cyclic) dipeptide, under the catalysis of the putative NRPS EAL92291. Like GliP, EAL92291 has a trimodular architecture (A_1-T_1-C_1-A_2-T_2-C_2-T_3) (Balibar & Walsh, 2006). This is in contrast to that of SirP of the sirodesmin cluster, and FtmPS for the formation of *cyclo*-L-Trp-L-Pro from the fumitremorgin cluster, as these do not contain the third thiolation domain T_3 (Gardiner *et al.*, 2004; Maiya *et al.*, 2006). It has been proven by biochemical investigation that the A_1 and A_2 domains in GliP are responsible for the activation of L-Phe and L-Ser, respectively, which are then loaded onto the T_1 and T_2 domains of GliP, respectively. The linear enzyme-bond peptide L-Phe-L-Ser-T_2 was formed under the catalysis of the condensation domain C_1 (Balibar & Walsh, 2006). The roles of the second condensation domain C_2, and the third thiolation domain T_3, are unclear, although the results of mutational experiments have suggested that they are involved in the same process (Balibar & Walsh, 2006). It has been speculated that the subsequent reactions to gliotoxin, e.g. methylation or sulphur insertion, may occur while the linear dipeptide is still covalently bound to the NRPS GliP (Balibar & Walsh, 2006). The release of an intermediate in the biosynthesis could occur at some later step. This could also be the case for EAL92291. This means that an unmodified cyclic dipeptide with the prenylated tryptophan is probably not involved in the biosynthesis of the unknown compound discussed here. The putative methyltransferase EAL92290 is probably responsible for the *N*-methylation of an enzyme-bound linear dipeptide or the diketopiperazine ring, similar to the proposed function of GliM and SirM in the biosynthesis of gliotoxin and sirodesmin (Gardiner & Howlett, 2005). The putative cytochrome P450 EAL92287 could be involved in the modification of the enzyme-bond dipeptide or a derivative thereof. The two regulators EAL92289 and EAL92294 are

probably involved in the regulation, and the putative transporter EAL92293 could be responsible for the efflux, of the product of the gene cluster from the fungus. Deletion of the genes from the genome of *N. fischeri* or *A. terreus*, in which this gene cluster is expressed, would explore the natural role of the gene cluster.

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BIOTECHNOLOGICALLY RELEVANT ENZYMES AND PROTEINS

Potential of a 7-dimethylallyltryptophan synthase as a tool for production of prenylated indole derivatives

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Abstract Recently, a gene for a 7-dimethylallyltryptophan synthase (7-DMATS) was identified in *Aspergillus fumigatus* and its enzymatic function was proven biochemically. In this study, the behaviour of 7-DMATS towards aromatic substrates was investigated and compared with that of the 4-dimethylallyltryptophan synthase FgaPT2 from the same fungus. In total, 24 simple indole derivatives were tested as potential substrates for 7-DMATS. With an exception of 7-methyltryptophan, all of the substances were accepted by 7-DMATS and converted to their prenylated derivatives, indicating a more flexible substrate specificity of 7-DMATS in comparison to that of FgaPT2. The relative activities of 7-DMATS towards these substrates were from 4% to 89% of that of L-tryptophan, much higher than that of FgaPT2. Structural elucidation of the isolated enzymatic products by nuclear magnetic resonance and mass spectrometry analysis proved unequivocally the prenylation at position C7 of the indole ring. Overnight incubation with eight substances showed that the conversion ratios were in the range of 55.9% to 99.7%. This study provided an additional example that prenylated indole derivatives can be effectively produced by using the overproduced and purified 7-DMATS.

Keywords *Aspergillus fumigatus* · Chemoenzymatic synthesis · 7-dimethylallyltryptophan synthase · Prenylated indole derivatives · Prenyltransferase · Prenylation

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Introduction

About one third of the clinically used drugs are natural products or natural product-derived molecules (Clardy and Walsh 2004; Newman and Cragg 2007). Structural modification of natural products are often necessary in drug development, e.g. for improvement in efficacy and pharmacokinetics (Walsh 2003). However, the complexity of many natural products, e.g. functional groups to be protected and the limited stereoselectivity of many chemical reactions, can limit the scope for making chemical modifications. In the last years, modifications of such molecules have been achieved by combinatorial biosynthesis, mutasynthesis or chemoenzymatic synthesis. Combinatorial biosynthesis was defined as the application of genetic engineering to modify biosynthetic pathways of natural products in order to produce new and altered structures using nature's biosynthetic machinery (Floss 2006). Mutasynthesis or mutational biosynthesis combines genetic and chemical approaches and was successfully used in a number of bacterial systems to produce biologically active substances (Galm et al. 2004; Weissman 2007; Weist and Süssmuth 2005). Chemoenzymatic synthesis is an in vitro approach with the help of purified proteins and was used successfully in the synthesis of peptides (Grunewald and Marahiel 2006), glycopeptides (Buskas et al. 2006), nucleotide deoxysugars (Rupprath et al. 2005), glycan libraries (Blixt and Razi 2006) and glycosides (Langenhan et al. 2005). Availability of genome sequences of bacteria and fungi, which has been increased tremendously in the last years (Jones 2007; Raskin et al. 2006), accelerates the identification of genes and enzymes involved in the biosynthesis of natural products (Abe et al. 2006; Keller et al. 2005; Li and Unsöld 2006) and therefore provides basis for use of these approaches. For a successful application of

these approaches, substrate flexibility of the biosynthetic enzymes is the prerequisite.

Our research interest focuses on the biosynthesis of prenylated indole derivatives from fungi. In general, prenylated indole derivatives belong to hybrid natural products containing both aromatic and isoprenoid moieties, which are widely distributed in plants, fungi and bacteria (Botta et al. 2005b). Prenylated indole derivatives from fungi carry diverse structures, show different biological activities and are found especially in the genera of *Claviceps*, *Aspergillus* and *Penicillium* (Stocking et al. 2000; Williams et al. 2000). Prenylation represents a critical step in the biosynthesis of these natural products of mixed biosynthetic origins. In the last years, progress has been achieved, especially, on the soluble prenyltransferases from bacteria and fungi, which catalyse the attachment of a prenyl moiety to an aromatic nucleus, known as "aromatic prenyltransferases". Examples of such enzymes from bacteria are the members of the ABBA family of aromatic prenyltransferases (Tello et al. 2008): CloQ in the biosynthesis of clorobiocin, (Pojer et al. 2003), Orf2 (renamed NphB) in the biosynthesis of naphterpin (Kuzuyama et al. 2005; Tello et al. 2008) and Fnq26 in the biosynthesis of a prenylated naphthaquinone (Haagen et al. 2007). We have identified five different prenyltransferases from *Aspergillus fumigatus*, which catalyse different prenyl transfer reactions to different positions of indole derivatives (Grundmann and Li 2005; Kremer et al. 2007; Ruan et al. 2008; Unsöld and Li 2005, 2006; Yin et al. 2007). Some of these soluble prenyltransferases show broad substrate specificities: e.g. NphB from *Streptomyces* sp., CL 190 and Fnq26 from *Streptomyces cinnnamonensis* catalyse the conversion of some dihydroxynaphthalenes to their geranylated derivatives (Haagen et al. 2007; Kuzuyama et al. 2005). NphB accepted even flavonoids as substrates. Similar phenomena have also been observed with fungal prenyltransferases: the brevianamide F prenyltransferase FtmPT1 and the cyclic dipeptide *N*-prenyltransferase CdpNPT accepted all of the tested tryptophan-containing cyclic dipeptides as substrates (Grundmann and Li 2005; Ruan et al. 2008; Yin et al. 2007). This tolerance towards aromatic substrates makes the enzymes especially attractive for their use as tools for chemoenzymatic synthesis, because prenylation represents not only a critical step in the biosynthesis of these compounds, but also often results in the formation of biologically active substances (Sanz-Cervera et al. 2000; Zhao et al. 2002). Therefore, chemoenzymatic synthesis using prenyltransferases could be used as a novel strategy for the synthesis of known compounds of emerging pharmaceutical interest and could create chemical libraries of novel prenylated compounds for biological screenings (Botta et al. 2005a).

Investigation of the substrate specificity of the 4-dimethylallyltrytophan synthase FgaPT2 from *A. fumigatus* showed that 16 of 24 commercially available simple indole derivatives were accepted as substrates, in addition to its natural substrate L-tryptophan (Steffan et al. 2007). These compounds carry modifications at the indole moiety or at the side chain. Interestingly, indole derivatives lacking the amino or carboxyl group at the side chain were also accepted by FgaPT2 (Steffan et al. 2007). Eight substances were selected for chemoenzymatic synthesis and found to be converted to their prenylated derivatives with conversion ratios of up to 99.7% (Steffan et al. 2007). This finding encouraged us to investigate the substrate specificity of another dimethylallyltryptophan synthase, 7-dimethylallyltryptophan synthase (7-DMATS), from the same fungus (Kremer et al. 2007), because some simple prenylated indole derivatives were reported to be biologically active, e.g. flustramines with cytotoxic properties (Lysek et al. 2002). In contrast to the prenylation of tryptophan at position C4 of the indole moiety catalysed by FgaPT2, 7-DMATS catalyses the prenylation at position C7. Therefore, two different prenylated derivatives could be obtained from one substrate by using FgaPT2 and 7-DMATS. Here we report the substrate specificity of 7-DMATS and its potential to be used for production of prenylated indole derivatives.

Materials and methods

Chemicals

Trisammonium salt of dimethylallyl diphosphate (DMAPP) was synthesised in analogy to the synthesis of trisammonium geranyl diphosphate reported by Woodside et al. (1988). The other substrates were obtained from Sigma, Aldrich, Fluka, Bachem, Roth, Acros and Alfa Aesar, respectively, and of the highest purity available.

Cloning of Afua_3g12930, overproduction and purification of His_6-7-DMATS

The coding region of Afua_3g12930 was amplified using PCR from cDNA of *A. fumigatus* strain B5233 which is available in the form of phagemids isolated from a cDNA library and cloned into the cloning vector pGEM-T. For gene expression the coding sequence of Afua_3g12930 was released from pGEM-T and cloned into the vector pQE60 resulting in the expression plasmid pLW40 (Kremer et al. 2007).

Escherichia coli XL1 Blue MRF' cells harbouring pLW40 were induced by 0.8 mM of isopropyl-beta-D-thiogalactopyranoside at 37°C. His_6-7-DMATS was puri-

fied with Ni-NTA agarose to apparent homogeneity as judged by sodium dodecyl sulfate polyacrylamide gel electrophoresis and a protein yield of 5 mg of purified His_6-tagged 7-DMATS per litre of culture was obtained (Kremer et al. 2007). The observed molecular weight was 50 kDa and corresponded well to the calculated value of 54 kDa for His_6-7-DMATS.

Determination of molecular weight of native His_6-7-DMATS

The molecular weight of native His_6-7-DMATS was determined by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Health Care, Freiburg, Germany) which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2,000 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa) and aprotinin (6.5 kDa) (GE Health Care, Freiburg, Germany). The proteins were eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The molecular weight of native His_6-7-DMATS was determined as 65 kDa. This proved that native 7-DMATS acts as a monomer.

Stability of the overproduced and purified His_6-7-DMATS

The purified His_6-7-DMATS was relatively stable under the conditions used for enzyme assays. More than 80% of original activity was recovered after preincubation at 37°C in the absence of the substrates for 16 h.

Conditions for enzymatic reactions

All of the enzyme assays contained 50 mM Tris-HCl (pH 7.5) and 10 mM $CaCl_2$. They differed from each other by incubation volumes, amounts of 7-DMATS and incubation times. The reaction mixtures of the standard assay for determination of the substrate specificity (100 µl) contained 1 mM tryptophan or derivatives, 1 mM DMAPP and 0.6 µM of purified His_6-7-DMATS. After incubation for 1 h at 37°C, the reaction was stopped with 10 µl of trichloroacetic acid (1.5 M). After removal of the protein by centrifugation (15,000×*g*, 10 min, 4°C), the enzymatic products were analysed on a high performance liquid chromatography (HPLC) system described below. Two independent assays were carried out routinely for quantification. The assays for isolation of the enzymatic products for structural elucidation (5 ml) contained 1 mM tryptophan or derivatives, 2 mM DMAPP and 1.6 µM of purified His_6-7-DMATS and were incubated for 16 h. The reaction mixtures were concentrated on a rotation evaporator at 30°C to a volume of 700 µl before injection. The assays for chemoenzymatic synthesis (100 µl) contained 1 mM tryptophan or derivatives, 2 mM DMAPP and 50 µM of purified His_6-7-DMATS and were incubated for 16 h.

HPLC analysis and determination of the conversion rate

Reaction mixtures of 7-DMATS were analysed on an Agilent HPLC Series 1100 by using an Eclipse XDB-C18 column (4.6×150 mm, 5 µm, Agilent) at a flow rate of 1 ml min^{-1}. Water (solvent A) and acetonitrile (solvent B), each containing 0.5% (*v*/*v*) trifluoroacetic acid, were used as solvents. A gradient was run from 15% to 70% B in 15 min. After washing with 100% solvent B for 5 min, the column was equilibrated with 15% solvent B for 5 min. The substances were detected with a photo diode array detector.

In general, the conversion rate was defined as the decrease of the peak area of the substrate in the HPLC chromatogram at 277 nm. For this purpose, the peak area of the substrate was calculated from data obtained by injection of 10 nmol instead of 100 nmol substrate used in the enzyme assays, to ensure the linearity of the analysis. For substrates with low conversion rates, e.g. 2% or lower, the ratio of the peak areas of product to substrate was used.

Spectroscopic analysis

The isolated products (50–200 µg) were analyzed by ^{1}H-nuclear magnetic resonance (NMR) spectroscopy, homonuclear correlation spectroscopy (H-H-COSY) as well as by positive and negative electrospray ionization (ESI) mass spectrometry with a ThermoFinnigan TSQ Quantum. The mass spectrometer was coupled with an Agilent HPLC series 1100 equipped with a RP18-column (2×250 mm, 5 µm). For separation, the column was run with 10% solvent B (methanol) in solvent A (water), each containing 0.1% of formic acid for 5 min, followed by a gradient from 10% to 100% B over 30 min. After washing with 100% B, the column was equilibrated with 10% B for 10 min. The flow rate was at 0.2 ml min^{-1}.

NMR spectra were taken on an Avance DRX 500 spectrometer (Bruker) using 0.1 M DCl as solvent. The δ values are given in ppm and coupling constants in Hz. The solvent signal at 4.81 ppm was used as reference. NMR and MS data are given in Tables 3, 4 and 5, respectively.

Results

7-DMATS accepted, with an exception of 7-methyltryptophan, all of the tested simple indole derivatives as substrates

In the course of our investigations on prenyltransferases, we have identified a putative prenyltransferase gene Afua_

3g12930 on chromosome 3 of the pathogenic fungus *A. fumigatus* Af293. The gene Afua_3g12930, which encodes for a 7-dimethylallyltryptophan synthase 7-DMATS (= EAL92290), belongs to a putative biosynthetic gene cluster consisting probably of eight putative genes: two putative transcription factor genes Afua_3g12940 and Afua_3g 12890, one putative transporter gene (Afua_3g12900), one gene with unknown function (Afua_3g12950) and four biosynthetic genes, i.e. the non-ribosomal peptide synthetase gene Afua_3g12920, the prenyltransferase gene Afua_3g12930 described in this study, Afua_3g12960 a gene coding for a putative cytochrome P450 enzyme and a putative methyltransferase gene Afua_3g12910. Homologous clusters were also found in the genome sequences of *Aspergillus terreus* NIH2624 and *Neosartorya fischeri* NRRL181 (Kremer et al. 2007). The end product of this cluster is unknown (Cramer Jr. et al. 2006; Nierman et al. 2005). Afua_3g12920 is not or very low expressed in its native host (Cramer Jr. et al. 2006) and no data are available on the expression of Afua_3g12930. Our previous study showed that 7-DMATS catalyses the prenylation of tryptophan at position C7 (Kremer et al. 2007). To extend our knowledge on the substrate specificity, 24 simple indole derivatives were incubated with 7-DMATS under the standard assay condition (Materials and methods). With an exception of 7-methyltryptophan, product formation could be observed for all of the substrates in HPLC chromatograms (data not shown). Liquid chromatography–mass spectrometry (LC–MS) analysis of the reaction mixtures confirmed the presence of the monoprenylated derivatives. For better comparison with FgaPT2 (Steffan et al. 2007), the tested compounds are grouped in two categories (Tables 1 and 2).

The members of group I are tryptophan derivatives with modifications at the side chain, i.e. indole derivatives with different substitutions at position C3. All of the 14 compounds of this group tested in this study were accepted by 7-DMATS, with conversion ratios of about 4% to 89% of that of L-tryptophan. These results showed that 7-DMATS has a somehow broader substrate specificity than FgaPT2, which accepted 11 of 14 substances from this group (Steffan et al. 2007). Similar to results obtained from FgaPT2 (Steffan et al. 2007), the configuration at C11 is also important for the enzymatic activity of 7-DMATS. A relative yield of 11.8% of L-tryptophan, comparable to that of FgaPT2, could be detected with D-tryptophan (Table 1). This value is slightly lower than that determined in the previous study of 15.5% (Kremer et al. 2007), which is probably caused by the different conditions, e.g. different substrate concentrations, incubation times and amounts of the purified enzyme. Some of the tested substances were commercially available only as mixtures of D- and L-enantiomers. If a similar behaviour of the enzyme towards all of the tested substances is assumed (stereochemistry at the position C11), it can be expected that the L-form was accepted better than its D-form. However, an exact conversion ratio can not be predicted or detected in our experiments.

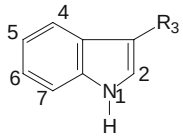
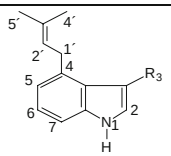
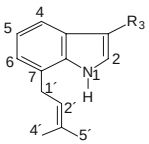
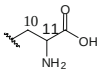
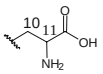
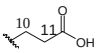
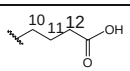
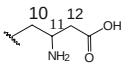
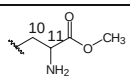
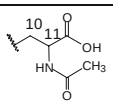
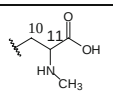
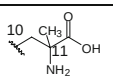
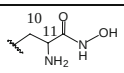
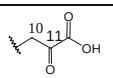
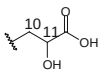
Modification of the side chain of tryptophan resulted in decreasing of relative enzymatic activity. E.g. deamination of tryptophan to indole-3-propionic acid resulted in a significantly reduced relative activity of 8.5% (Table 1), clearly lower than that observed with FgaPT2 with a relative activity of 32.2% of that of L-tryptophan. Interestingly, comparable activity to that of indole-3-propionic acid was observed for indole-3-acetic acid and indole-3-butyric acid at 10.8% and 9.1% of that of L-tryptophan, respectively. It seems that slight change of the chain length did not play an important role for an acceptance by 7-DMATS. This finding is in contrast to the results obtained from FgaPT2, for which a complete loss of enzymatic activity was observed after shortening the side chain to indole-3-acetic acid or extending to indole-3-butyric acid (Steffan et al. 2007). Additional modifications at C11 of indole-3-propionic acid by hydroxylation to indole-3-lactic acid and by hydroxylation and oxidation to indole-3-pyruvic acid reduced the enzyme activity to about half of that of indole-3-propionic acid (Table 1).

Decarboxylation of L-tryptophan to tryptamin, methylation to L-tryptophan methyl ester or acetylation of the 11-amino moiety to *N*-acetyltryptophan resulted in decreased, but detectable and comparable enzyme activities of about 7% of that of L-tryptophan (Table 1). Hydroxymation of tryptophan resulted in much stronger reduction of enzyme activity (Table 1).

Significant enzyme activity of 82.2% and 28.2% of that of L-tryptophan, respectively, could be detected with L-abrine and L-ß-homotryptophan as substrates. This finding is in consistence with their high conversion rates by FgaPT2 (Steffan et al. 2007). Interestingly, 11-methyl-DL-tryptophan, which was accepted by FgaPT2 with a relative activity of only 1.6% of that of L-tryptophan, was very well accepted by 7-DMATS, with a relative activity of 19.1% of that of L-tryptophan. As conclusion, the retention of the free carboxyl and amino group of tryptophan is also important for the enzyme activity of 7-DMATS. Methylation at the amino group or at C11 as well as insertion of a carbon atom between amino and carboxyl groups showed lower effect on the enzyme activity than the decarboxylation, deamination, esteration, hydroxymation or acetylation of tryptophan.

The members of group II (Table 2) consist of tryptophan and its 10 derivatives with modifications at the indole ring. With an exception of 7-methyltryptophan, all of the tested compounds were accepted by 7-DMATS (Table 2). Methylation at the indole ring resulted in reduced, but still significant product formation. In comparison to that of L-tryptophan, relative yields of 35.8%, 89.4%, 74.8% and

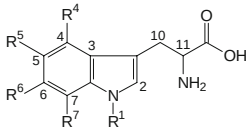
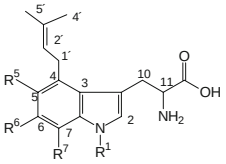
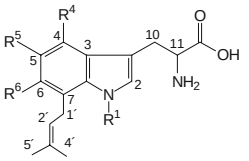
Table 1 Comparison of the relative substrate specificity of 7-DMATS with that of FgaPT2 (Steffan et al. 2007) towards tryptophan derivatives with modifications at the side chain

Substrate		Products	
			
R^3=		rel. yield [%] FgaPT2	rel. yield [%] 7-DMATS
L-tryptophan		100.0	100.0
D-tryptophan		9.7	11.8
indole-3-propionic acid		32.2	8.5
indole-3-acetic acid		< 0.2	10.8
indole-3-butyric acid		< 0.2	9.1
L-β-homotryptophan		51.5	28.2
tryptamin		4.6	7.5
L-tryptophan methyl ester		7.2	7.2
N-acetyl-DL-tryptophan		< 0.2	7.7
L-abrine (N_α-methyl-L-tryptophan)		58.0	82.2
11-methyl-DL-tryptophan		1.6	19.1
L-tryptophan hydroxamate		22.4	4.6
indole-3-pyruvic acid		29.4	4.8
DL-indole-3-lactic acid		10.5	4.3

In this experiment, the conversion rate of L-tryptophan was found to be 41.3%, determined as described in "Materials and methods". The relative yield or relative activity was defined as the ratio of the conversion rate of a substrate to that of L-tryptophan

19.8% were observed for 1-, 4-, 5-, and 6-methyltryptophan (Table 2), respectively, which is significantly higher than those of FgaPT2 in the range of 8.1% to 21% of L-tryptophan. As observed for FgaPT2, the efficiency of the prenylation reduced progressively with the decreased distance of the methyl group to the prenylation position (Steffan et al. 2007). The relative yields of the racemic mixtures of 4-methyltryptophan and 5-methyltryptophan

Table 2 Comparison of the relative substrate specificity of 7-DMATS with that of FgaPT2 (Steffan et al. 2008) towards tryptophan derivatives carrying modifications at the indole ring

Substrate						Products	
							
	R^1	R^4	R^5	R^6	R^7	rel. yield [%] FgaPT2	rel. yield [%] 7-DMATS
L-tryptophan	H	H	H	H	H	100	100
D-tryptophan	H	H	H	H	H	9.7	11.8
1-methyl-L-tryptophan	CH_3	H	H	H	H	0.4	35.8
4-methyl-DL-tryptophan	H	CH_3	H	H	H	< 0.2	89.4
5-methyl-DL-tryptophan	H	H	CH_3	H	H	8.1	74.8
6-methyl-DL-tryptophan	H	H	H	CH_3	H	15.2	19.8
7-methyl-DL-tryptophan	H	H	H	H	CH_3	16.3	< 0.3
5-hydroxy-L-tryptophan	H	H	OH	H	H	21.0	26.7
5-methoxy-DL-tryptophan	H	H	OCH_3	H	H	0.6	13.3
5-bromo-DL-tryptophan	H	H	Br	H	H	< 0.2	7.2
5-fluoro-L-tryptophan	H	H	F	H	H	< 0.2	10.7
6-fluoro-DL-tryptophan	H	H	H	F	H	< 0.2	33.7

For descriptions see Table 1

were higher than 55.9%, which could be calculated by a complete conversion of the L-forms and 11.8% of the L-forms as determined with D-tryptophan. This indicates that methylation at the indole ring could decrease the preference of the enzyme for L- over D-configured substrates. As expected for a 7-dimethylallyltryptophan synthase, no product could be detected with 7-methyl-DL-tryptophan, which confirmed the regioselectivity of the enzymatic reaction. Similar relative activities towards 5-hydroxy-L-tryptophan were observed with 7-DMATS and FgaPT2 at 26.7% and 21.0% of L-tryptophan, respectively. Similar to FgaPT2, methylation of this hydroxy group resulted in reduction of the enzymatic activity. 13.3% of that of L-tryptophan was observed for 5-methoxy-DL-tryptophan with 7-DMATS (Table 2). In contrast to the results obtained by using FgaPT2, halogenation at the indole ring resulted in reduced, but still significant enzyme activity of 7-DMATS. With 5-bromo-, 5-fluoro- and 6-fluoro-tryptophan, which were not accepted by FgaPT2 (Steffan et al. 2007), relative activities of 7.2%, 10.7% and 33.7% of that of L-tryptophan were detected.

7-DMATS catalyses the prenylation at position C7

Our previous results proved unequivocally the prenylation of tryptophan at position C7 (Kremer et al. 2007). To confirm the prenylation position, enzymatic products of 10 additional simple indole derivatives were isolated on a preparative scale and subjected to NMR and MS analysis. If necessary, the correlation of the protons was proven by H-H-COSY spectra. Comparison of the ^{1}H-NMR spectra of the isolated products (Tables 3 and 4) with the respective substrates revealed the disappearance of signals for one aromatic proton at position C4 or C7 and the presence of signals for a dimethylallyl moiety at 5.2–5.5 (t, H-2′), 3.5–3.6 (d, H_2-1′) and 1.7–1.9 ppm (H-4′ and H-5′), respectively. This indicated that the prenylation has taken place at position C4 or C7 of the indole moieties. Comparison of the spectra of the isolated compounds with those of FgaPT2 showed that the products of 7-DMATS differed clearly from those of the 4-prenylated derivatives obtained by using FgaPT2 (Steffan et al. 2007). Therefore, the structures of 7-DMA-*β*-homotryptophan, 7-DMA-abrine, *N*-

Table 3 ^{1}H-NMR data of 7-prenylated tryptophan derivatives with modifications at the side chain

	7-DMAT	7-DMA-abrine	7-DMA-ß-homotryptophan	*N*-acetyl-7-DMAT	11-methyl-7-DMAT
H-2	7.32, s	7.33, s	7.31, s	7.24, s	7.30, s
H-4	7.53, d, 7.6	7.52,d, 7.9	7.51, d, 7.9	7.50, d, 7.5	7.51, d, 6.9
H-5	7.14, t, 7.9	7.13, t, 7.9	7.13, t, 7.3	7.10, m	7.12, m
H-6	7.10, d, 8.2	7.10, d, 7.9	7.09, d, 7.3	7.06, m	7.09, d, 6.9
H-10	3.52, dd, 15.5, 5.4 3.43, dd, 15.5, 7.0	3.62, dd, 15.0, 4.2 3.52, dd, 15.0, 5.3	2.83, m 2.70, m	3.35, dd, 15.0, 5.4 3.23, dd, 15.0, 7.8	3.53, d, 15.3 3.32, d, 15.3
H-11	4.39, t, 6.1	4.30, t, 6.0	3.96, t, 7.0	4.69, t, 6.3	CH_3: 1.67, s
H-12	–	–	3.18, m	–	–
	–	N-CH_3: 2.73, s	–	$COCH_3$: 1.98, s	–
H-1′	3.58, d, 7.0	3.59, d, 7.2	3.58, d, 7.0	3.57, d, 6.6	3.58, d, 7.3
H-2′	5.46, br t, 7.0	5.47, t, 7.2	5.46, t, 7.0	5.46, t, 6.6	5.46, t, 7.3
H-4′	1.72, s	1.73, s	1.72, s	1.72, s	1.72, s
H-5′	1.74, s	1.75, s	1.74, s	1.74, s	1.74, s

For structures and numbering please see Table 1. The spectra were taken in 0.1 M DCl. The chemical shifts are given in ppm and coupling constants in Hz

acetyl-7-DMAT, 11-methyl-7-DMAT, 1-methyl-7-DMAT, 4-methyl-7-DMAT, 5-methyl-7-DMAT, 6-methyl-7-DMAT, 5-bromo-7-DMAT and 6-fluoro-7-DMAT could be identified unequivocally as prenylated derivatives of the respective substrates and the prenylation has taken place at position C7 of the indole rings (Tables 1 and 2). These results were also confirmed by LC–MS analysis (Table 5).

7-DMATS as a tool for chemoenzymatic synthesis

To test the potential of 7-DMATS for chemoenzymatic synthesis, eight selected substrates were incubated over night to determine the conversion rates. HPLC-chromatograms of the incubation mixtures are illustrated in Fig. 1.

Under the condition described in the "Materials and methods", L-tryptophan, L-abrine and ß-homotryptophan were converted almost completely to their prenylated derivatives. Very high conversion yields from 83.7% to 95.0% were observed with 4-methyl-DL-tryptophan, 5-methyl-DL-tryptophan and 11-methyl-DL-tryptophan**.** Somehow lower, but still significant conversion yields of 64.6 and 55.9% were determined with 6-methyl-DL-tryptophan and 6-fluoro-DL-tryptophan, respectively. These results demonstrated clearly that the soluble prenyltransferase 7-DMATS prenylated the selected substrates with a high efficiency and could be used as an effective tool for the prenylation of indole derivatives at position C7 of the indole rings.

Discussion

Information on substrate specificity is a prerequisite for successful and efficient chemoenzymatic synthesis, which

Table 4 ^{1}H-NMR data of 7-prenylated tryptophan derivatives with modifications at the indole moiety

	1-methyl-7-DMAT	4-methyl-7-DMAT	5-methyl-7-DMAT	6-methyl-7-DMAT	5-bromo-7-DMAT	6-fluoro-7-DMAT
H-2	7.32, s	7.28, s	7.29, s	7.26, s	7.32, s	7.29, s
H-4	7.53, d, 7.6	CH_3: 2.67, s	7.34, s	7.44, d, 7.9	7.68, s	7.45, d, 7.7
H-5	7.14, t, 7.6	6.98, d, 7.5	CH_3: 2.39, s	7.04, d, 7.9	–	6.96, d, 7.7
H-6	7.10, d, 7.6	6.88, d, 7.5	6.96, s	CH_3: 2.40, s	7.20, s	–
H-10	3.52, dd, 15.1, 5.4 3.43, dd, 15.1, 7.0	3.81, dd, 15.7, 5.1 3.62, dd, 11.8, 4.5	3.50, dd, 15.0, 5.4 3.40, dd, 15.0, 7.5	3.49, dd, 15.4, 5.1 3.41, dd, 15.4, 7.1	3.45, dd, 15.1, 5.4 3.37, dd, 15.1, 7.0	3.48, dd, 15.9, 6.2 3.41, dd, 15.0, 5.6
H-11	4.39, t, 6.7	4.28, dd, 9.8, 5.1	4.38, t, 6.5	4.38, t, 6.7	4.35, t, 6.5	4.37, t, 7.5
H-1′	3.59, d, 7.0	3.54, d, 7.0	3.53, d, 7.2	3.59, d, 7.0	3.54, d, 7.1	3.53, d, 7.0
H-2′	5.47, t, 7.0	5.44, t, 7.0	5.46, t, 7.2	5.17, br t, 7.0	5.43, t, 7.1	5.36, t, 7.0
H-4′	1.72, s	1.72, s	1.73, s	1.67, s	1.72, s	1.66, s
H-5′	1.75, s	1.74, s	1.75, s	1.82, s	1.73, s	1.77, s

For structures and numbering see please Table 2. The spectra were taken in 0.1 M DCl. The chemical shifts are given in ppm and coupling constants in Hz

Table 5 MS-data of the enzymatic products of 7-DMATS

	Enzymatic product	M_r	ESI (+) m/z			ESI (−) m/z	
			$[M+H]^+$	$[2M+H]^+$	ms^2 of $[M+H]^+$ (rel. intensity)	$[M-H]^-$	ms^2 of $[M-H]^-$ (rel. intensity)
group I	7-DMA-β-homotryptophan	286.2	287.1	–	270.0 (37), 252.0 (10), 224.1 (25), 214.0 (100), 202.0 (34), 200.0 (13), 196.2 (14)	285.3	285.2 (100), 268.2 (37), 225.3 (17), 224.3 (85)
	N-acetyl-7-DMAT	314.2	315.2	629.2	297.1 (6), 272.9 (22), 269.0 (78), 259.0 (16), 256.1 (48), 255.0 (8), 246.9 (57), 228.9 (11), 227.0 (11), 198.1 (100)	313.6	313.3 (36), 272.2 (6), 271.2 (100), 116.0 (5)
	N_α-methyl-7-DMAT	286.2	287.1	573.1	255.9 (100), 187.9 (21)	285.3	285.2 (91), 254.1 (26), 210.2 (77), 184.2 (100)
	11-methyl-7-DMAT	286.2	287.1	573.1	287.1 (8), 286.3 (11), 281.6 (6), 232.3 (8), 223.8 (9), 201.9 9), 184.9 (6), 130.0 (100)	285.3	285.2 (61), 241.2 (17), 224.2 (17), 224.3 (100), 84.3 (61)
group II	1-methyl-7-DMAT	286.2	287.1	–	–	285.3	–
	4-methyl-7-DMAT	286.2	287.3	–	271.1 (100), 270.1 (61), 255.9 (22), 254.9 (17), 229.1 (29), 228.0 (21), 220.0 (7), 215.0 (36), 214.1 (27)	285.3	286.2 (42), 285.2 (18), 242.2 (7), 225.3 (9), 199.9 (100), 198.2 (37)
	5-methyl-7-DMAT	286.2	287.2	573.4	270.1 (100), 254.8 (25), 252.2 (9), 228.0 (44), 226.1 (23), 218.9 (6), 214.1 (43), 202.0 (10), 170.1 (14), 158.1 (10)	285.3	285.1 (73), 284.2 (6), 241.1 (26), 224.2 (61), 198.2 (100)
	6-methyl-7-DMAT	286.2	287.1	574.1	270.2 (100), 228.1 (5), 218.9 (5), 214.1 (6)	285.2	285.0 (100), 241.1 (6), 224.2 (6), 198.1 (10)
	5-bromo-7-DMAT	350.1/ 352.1	351.2/ 353.3	–	351.1 (100), 350.2 (26), 290.2 (81), 289.3 (11), 264.3 (54), 263.3 (6)	349.4/ 351.3	349.1 (100), 289.3 (8), 288.2 (71), 262.3 (44)
	5-fluoro-7-DMAT	290.3	291.1	582.0	222.9 (7), 205.9 (100)	289.2	289.2 (86), 245.2 (27), 228.2 (86), 202.2 (100)

became in the last years a new strategy for drug discovery and development. In comparison to chemical synthesis, chemoenzymatic synthesis is performed under mild conditions, e.g. at low incubation temperature and in aqueous solutions with nearly neutral pH values. Therefore, undesired reactions and rearrangements could be usually avoided. As shown in this study, it is not necessary to protect functional groups of the substrates for chemoenzymatic synthesis, which is usually essential for chemical synthesis. Furthermore, different protecting and deprotecting strategies must be often developed for similar reactants in the chemical synthesis. An additional aspect is the high regio- and stereoselectivity of chemoenzymatic synthesis, which is often difficult to be realized in the chemical synthesis.

The results described in this study showed the broad substrate specificity of a dimethylallyltryptophan synthase 7-DMATS from *A. fumigatus*. With the exception of 7-methyltryptophan, all of the 24 tested indole derivatives were accepted by this enzyme. These substrates also included substances such as halogenated tryptophan derivatives, which were not accepted by another dimethylallyltryptophan synthase FgaPT2 from the same fungus (Steffan et al. 2007), indicating a more flexible substrate specificity of 7-DMATS than that of FgaPT2. By comparison of relative activities of the substrates (Tables 1 and 2), it is obvious that the modifications at the indole ring showed lower effect on the enzyme activity of 7-DMATS than the modifications at the side chain of tryptophan.

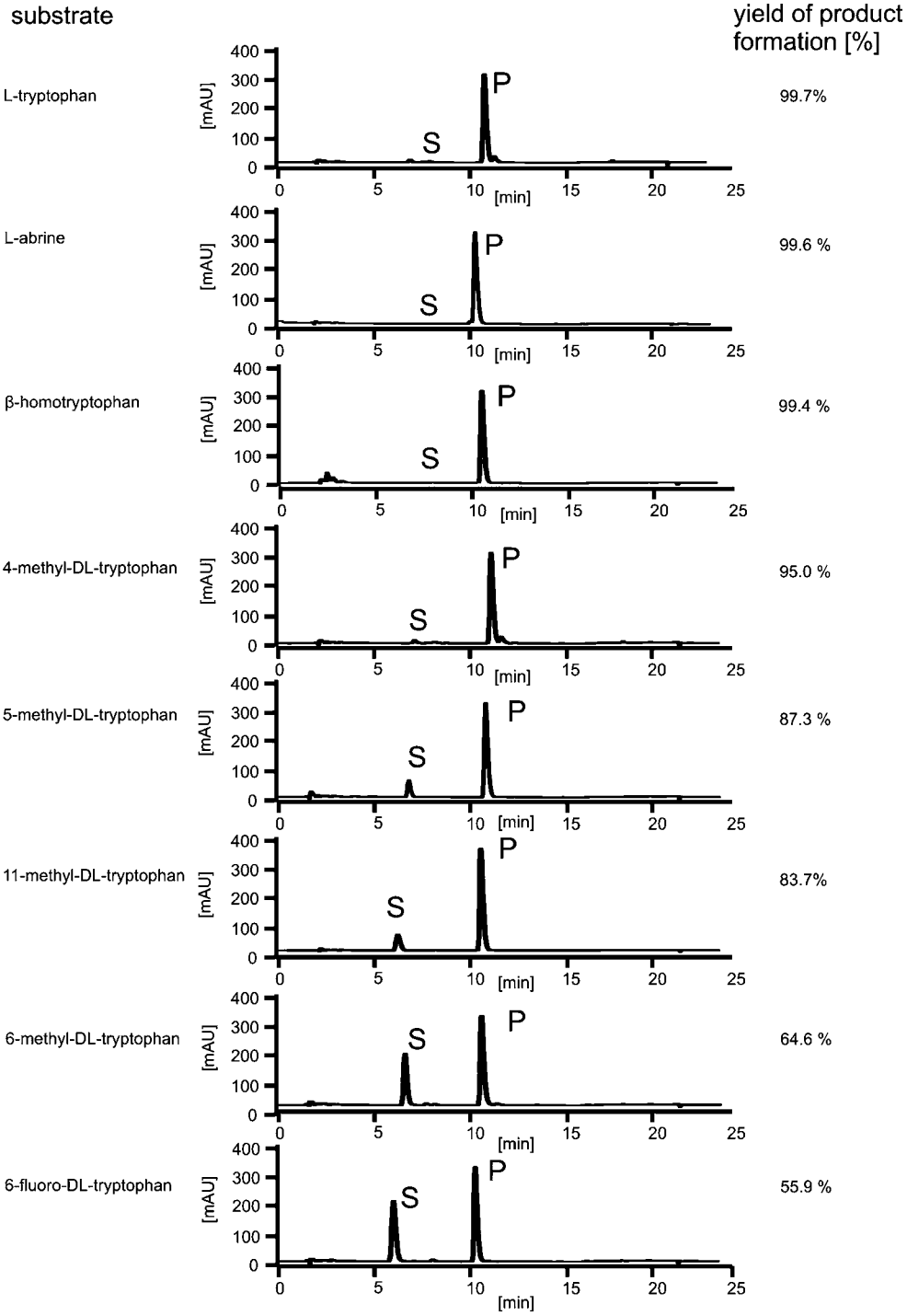


Fig. 1 HPLC analysis of the reaction mixtures of selected substrates with 7-DMATS after overnight incubation. Detection was carried out at 277 nm (*S* substrate, *P* product)

The non-acceptance of 7-methyltryptophan by 7-DMATS indicated the prenylation position at C7 of the indole moiety (Kremer et al. 2007) and the high regioselectivity of the enzyme towards simple tryptophan derivatives, which could be proven by isolation and structural elucidation of 11 enzymatic products. NMR and MS analysis (Tables 3, 4 and 5) revealed unequivocally the prenylation of these compounds at position C7 of the indole rings.

The results obtained with 7-DMATS are in consistence with those observed with other indole prenyltransferases from *A. fumigatus* (Grundmann and Li 2005; Ruan et al. 2008; Steffan et al. 2007; Yin et al. 2007). All of these enzymes accepted only DMAPP as prenyl donor, but showed high flexibility towards their aromatic substrates and catalysed regiospecific prenylations. E.g., both FtmPT1 and CdpNPT accepted the same tryptophan-containing cyclic dipeptides as substrates, but catalysed prenyl transfer

reactions at different positions of the indole moiety, i.e. FtmPT1 at C2 and CdpNPT at N1 (Grundmann and Li 2005; Ruan et al. 2008; Yin et al. 2007). Similar phenomenon was observed with FgaPT2 and 7-DMATS, which catalyse the prenylations of tryptophan at position C4 and C7, respectively. In summary, products with different prenylation patterns could be obtained from one indole derivative by using different prenyltransferases. This makes the enzymes in this group especially attractive for biotechnological production of active compounds.

Our study also exemplifies that the indole prenyltransferases could be conveniently used for production of different structures. Overnight incubation of eight substances with 7-DMATS showed that the conversion ratios were in the range of 55.9% to 99.7%. More importantly, these enzymes are soluble proteins and could be actively overproduced in *E. coli*. The development of soluble recombinant enzymes in a convenient system is one of the best solutions in terms of ease of manipulation, efficiency of catalysis and overall cost of the biotransformation. Such approaches offer the potential to significantly increase the structural diversity in drug discovery programmes.

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Tryptophan Aminopeptidase Activity of Several Indole Prenyltransferases from *Aspergillus fumigatus*

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SUMMARY

Recently, five indole prenyltransferases from *Aspergillus fumigatus* have been proven biochemically to be responsible for prenylations of diverse substrates. In this study, we show peptidase activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, with preference for linear peptides containing a tryptophanyl moiety at the N terminus. Testing of 31 peptides revealed that these enzymes shared similar substrate specificity and accepted H-L-Trp-L-Ala-OH and H-L-Trp-Gly-OH as best substrates for aminopeptidase activity. By using H-L-Trp-Gly-OH as substrate, K_m values at 350, 380, 300, and 420 μM and enzymatic rate constants k_{cat}/K_m at 0.51, 0.24, 0.53, and 0.14 $mM^{-1}s^{-1}$ were determined for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. In contrast to prenyltransferase activities, the aminopeptidase activities were strongly or completely inhibited by EDTA. Mn^{2+} increased the aminopeptidase activities of FtmPT1 and CdpNPT up to 4- and 6-fold, respectively. To the best of our knowledge, this is the first report on the catalytic promiscuity of prenyltransferases.

INTRODUCTION

From the genome sequence of *Aspergillus fumigatus* AF293, seven putative prenyltransferase genes could be identified by bioinformatic approach that share sequence similarity to the dimethylallyltryptophan synthase from *Claviceps purpurea* (Nierman et al., 2005). They belong probably to five different biosynthetic gene clusters of secondary metabolites (Nierman et al., 2005). Recently, we have characterized five of these genes from four biosynthetic gene clusters by gene cloning, expression, and biochemical investigation (Grundmann and Li, 2005; Kremer et al., 2007; Ruan et al., 2008; Unsöld and Li, 2005, 2006; Yin et al., 2007). Our results proved that these enzymes catalyze different prenylations at various positions of the indole rings of diverse secondary metabolites (Figure 1); e.g., FgaPT2 and FgaPT1 are involved in the biosynthesis of fumigaclavine C and catalyze the prenylation of L-tryptophan at position C4 and the prenylation of fumigaclavine A at position C2 of the indole rings, respectively (Figures 1A and 1E; Unsöld and Li, 2005, 2006). Therefore, FgaPT2 functions as a 4-dimethylallyltryptophan synthase. A second dimethylallyltryptophan synthase, 7-DMATS, catalyzes the prenylation of L-tryptophan at position C7 of the indole ring (Figure 1B; Kremer et al., 2007). From a gene cluster of fumitremorgins, FtmPT1 was proven to catalyze the prenylation of cyclo-L-Trp-L-Pro (brevianamide F) at position C2 of the indole ring (Figure 1C; Grundmann and Li, 2005). The very recently identified prenyltransferase, CdpNPT, was found to catalyze the reverse prenylation of tryptophan-containing cyclic dipeptides at position N1 of the indole rings (Figure 1D; Ruan et al., 2008). During the investigation on 7-DMATS, it was found out that this enzyme accepted also tryptophan-containing cyclic dipeptides and linear dipeptides such as H-L-Trp-Gly-OH as substrates (Kremer et al., 2007). Surprisingly, two product peaks were detected in the incubation mixture of H-L-Trp-Gly-OH, dimethylallyl diphosphate (DMAPP) and the recombinant His_6-7-DMATS (Figure 2A), but not in the control incubation with heat-inactivated protein (Figure 2B). These compounds were identified as H-L-7-DMAT-Gly and 7-dimethylallyltryptophan (7-DMAT) (Figure 1F) after preparative isolation and structural elucidation by NMR and MS analysis (unpublished data). The presence of 7-DMAT in the reaction mixture indicates a hydrolytic process after or before the prenylation reaction during the incubation, i.e., hydrolysis of the expected prenylated product H-L-7-DMAT-Gly to 7-DMAT or hydrolysis of the substrate H-L-Trp-Gly-OH to L-Trp, which was subsequently prenylated to 7-DMAT. In both cases, peptidase activity of the enzyme should be involved. To our knowledge, no peptidase activity of a prenyltransferase is described in the literature. In this study, we report on the aminopeptidase activities of the indole prenyltransferases from *Aspergillus fumigatus*.

RESULTS

Peptidase Activities of the Prenyltransferases

As shown in Figure 2, the substrate H-L-Trp-Gly-OH was chemically stable under the condition for enzymatic reactions and no additional peak was detected in the incubation mixtures with heat-denaturated recombinant proteins. HPLC analysis of the incubation mixture of H-L-Trp-Gly-OH and His_6-7-DMATS in the absence of DMAPP showed one product, which was absent in the control assay with heat-inactivated His_6-7-DMATS.

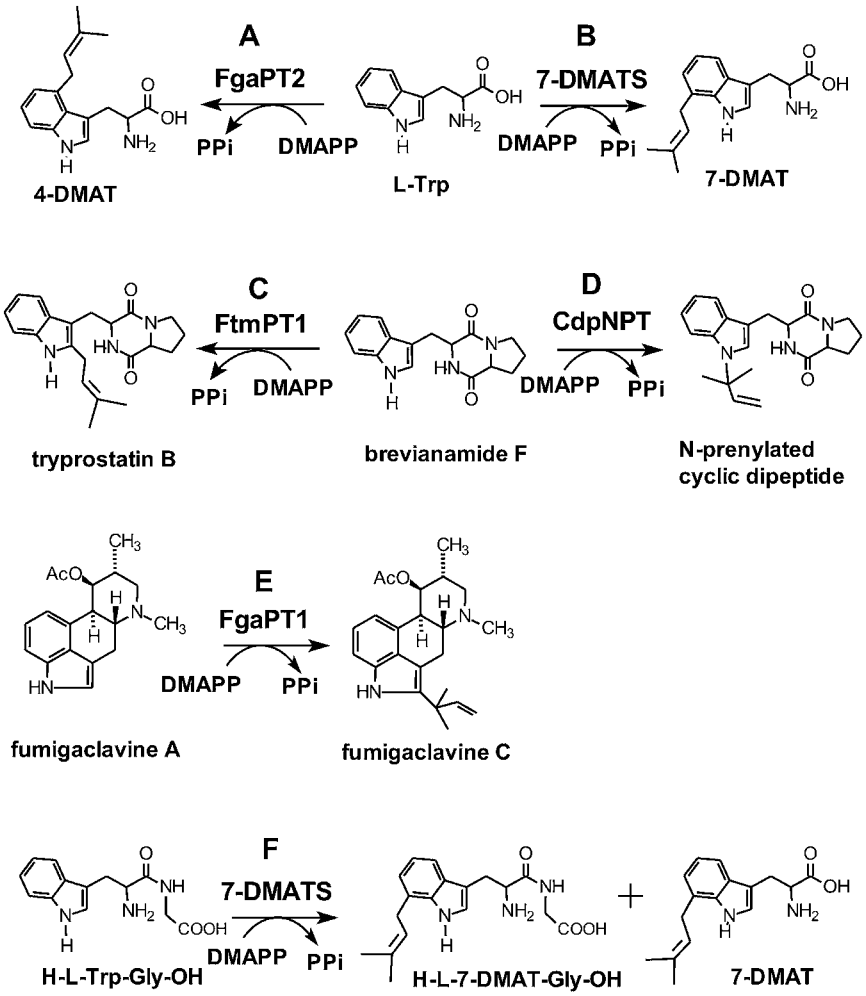

Figure 1. Prenyltransfer Reactions Catalyzed by Different Prenyltransferases from *Aspergillus fumigatus*

(A and B) Conversion of L-Trp to 4-dimethylallyltryptophan by FgaPT2 (A) and to 7-dimethylallyltryptophan by 7-DMATS (B).

(C and D) Conversion of brevianamide F by FtmPT1 to tryprostatin B (C) and to N-prenylated cyclic dipeptide by CdpNPT (D).

(E) Conversion of fumigaclavine A to C by FgaPT1.

(F) Conversion of H-L-Trp-Gly-OH to H-L-7-DMAT-Gly-OH and 7-dimethylallyltryptophan by 7-DMATS.

Incubation with 0.6 μM protein for 16 hr resulted in complete conversion of H-L-Trp-Gly-OH to the enzymatic product (Figure 2C). The enzymatic product could be identified as L-Trp by cochromatography on HPLC and by LC-MS analysis. Positive ESI-LC-MS analysis of the reaction mixture indicated the presence of $[M+1]^+$ for tryptophan at m/z 205 and $[M+1]^+$ for glycine at m/z 76. This result proved that the indole prenyltransferase 7-DMATS showed indeed a peptidase activity and hydrolysis of the dipeptide H-L-Trp-Gly-OH to the amino acids tryptophan and glycine has taken place during the incubation. To prove whether other purified and identified indole prenyltransferases also shared this property, peptidase activities of FgaPT1, CdpNPT, FtmPT1, and FgaPT2 were tested by using H-L-Trp-Gly-OH as substrate in the presence of Ca^{2+}, but in the absence of DMAPP. As shown in Figure 2, product formation was clearly detected with recombinant FgaPT1, which is absent in the negative control (Figures 2E and 2F). A conversion rate of 63% could be achieved after incubation for 16 hr. In the presence of 5 mM of Ca^{2+}, CdpNPT and FtmPT1 showed lower activities than 7-DMATS and FgaPT1, with conversion rates of 17.7 and 10.3%, respectively (Figures 2G and 2K). On the other hand, another dimethylallyltryptophan synthase FgaPT2 showed no detectable peptidase activity under this condition (Figures 2O and 2P). These data proved that FgaPT1, CdpNPT, and FTmPT1 are also active as peptidases.

Influence of Metal Ions and ATP on the Peptidase Activities

Our previous results on the prenyltransfer activities of the prenyltransferases showed that the enzymatic activities were not inhibited by addition of the metal chelating agent EDTA (Grundmann and Li, 2005; Kremer et al., 2007; Unsöld and Li, 2005; Unsöld and Li, 2006; Yin et al., 2007), indicating that metal ions were not essential for the prenyltransfer reactions, although Ca^{2+} ions enhance the reactions of some enzymes in this group. Investigation of the peptidase activity of 7-DMATS by using H-L-Trp-Gly-OH as substrate showed that no significant change of the enzyme activity was observed by addition of divalent metal ions such as Ca^{2+}or Mg^{2+} to a final concentration of 5 (Table 1) or 10 mM (data not shown). It seems that metal ions were also not essential for the peptidase activity. In the incubation mixture with EDTA, however, the peptidase activity of 7-DMATS was reduced to 11.6% of that of the incubation mixture without additives. This indicated strongly that divalent metal ions were important for the enzymatic activity. Similar results were also obtained for the peptidase activity of FgaPT1, FtmPT1 and CdpNPT: no or only slight increasing (150% or less) of enzyme activities was observed by addition of metal ions such as Ca^{2+} and Mg^{2+}. However, more than 50% inhibition of the peptidase activity of FgaPT1 was detected after addition of EDTA to the incubation mixture. Peptidase activities of CdpNPT and FtmPT1 were completely inhibited by 5 mM EDTA (Table 1). To explain this contra verse phenomenon, dependence of peptidase activities of 7-DMATS, FgaPT1, FtmPT1, and CdpNPT on additional metal ions was investigated. The enzymatic activities of 7-DMATS and FgaPT1 were reduced in the assays with Mn^{2+} to 84 and 70% of those of incubations without additives, respectively. In contrast, the peptidase activities of FtmPT1 and CdpNPT have been significantly increased in the presence of Mn^{2+} up to four- and six-fold, respectively. These data proved that Mn^{2+} is essential for the peptidase activities

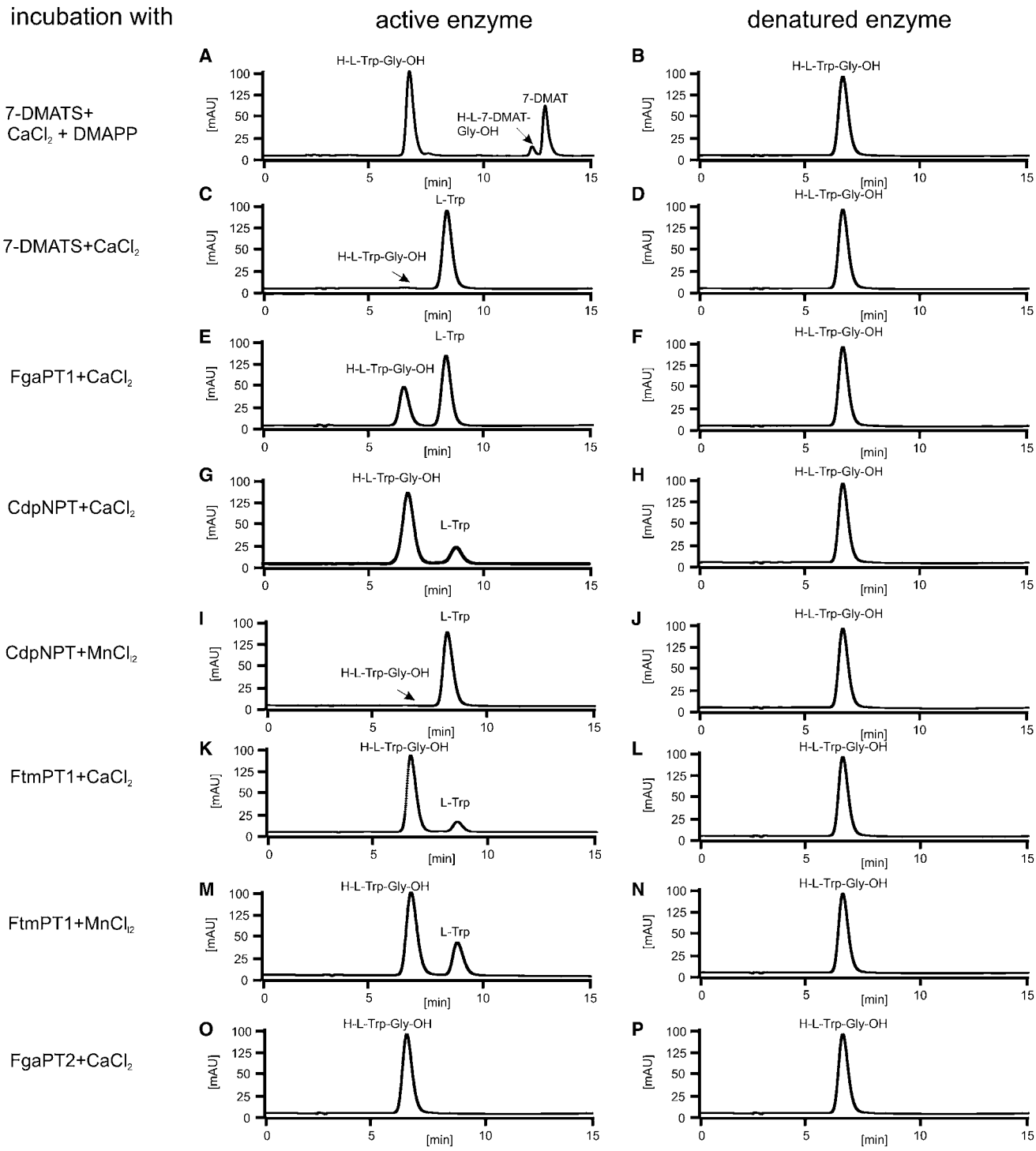

Figure 2. Hydrolysis of H-L-Trp-Gly-OH by Different Prenyltransferases under Different Conditions

(A–P) The incubation mixtures contained 2 mM substrate, 5 mM metal ions, and 0.6 μM 7-DMATS and FtmPT1 or 0.3 μM CdpNPT and FgaPT1 and were incubated at 37°C for 16 hr. Detection was carried out at 277 nm.

of these two enzymes. By using H-L-Trp-Gly-OH as substrate, FtmPT1 showed a maximum of peptidase activity in the presence of Mn^{2+} at a final concentration of 2.5 to 30 mM (data not shown). Incubation of CdpNPT with H-L-Trp-Gly-OH in the presence of Mn^{2+} at a final concentration of 5 mM for 16 hr resulted in complete conversion of the substrate to L-Trp (Figure 2I). A conversion rate of 28% could be achieved with FtmPT1 in the presence of Mn^{2+} (Figure 2M), approximately three times as

Table 1. Dependence of the Peptidase Activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 on the Presence of Different Additives with Final Concentration of 5 mM

	Relative Activity (%)				
Additive	7-DMATS	FgaPT1	CdpNPT	FtmPT1	FgaPT2
No additive	100 ± 4.1	100 ± 4.1	100 ± 0.8	100 ± 1.6	≤0.2
ATP	151.6 ± 4.4	145 ± 3.1	243.6 ± 5.8	149.4 ± 6.3	≤0.2
Ca^{2+}	102.7 ± 2.9	144.2 ± 8.5	69.2 ± 6.7	144.1 ± 2.3	≤0.2
Mg^{2+}	103.5 ± 1.2	137.9 ± 1.8	104.0 ± 6	155.2 ± 3.8	≤0.2
Mn^{2+}	84.0 ± 2.0	69.7 ± 1.1	591.2 ± 5.2	421.6 ± 1.7	≤0.2
Zn^{2+}	≤0.2	≤0.2	≤0.2	≤0.2	≤0.2
Co^{2+}	10.2 ± 1.3	32.3 ± 0.3	46.9 ± 1.5	130.4 ± 2.4	≤0.2
Fe^{2+}	≤0.2	≤0.2	34.2 ± 2.8	42.8 ± 0.5	≤0.2
Na^{+}	97.0 ± 3.9	108.2 ± 5.1	94.8 ± 0.8	108.6 ± 2	≤0.2
K^{+}	103.7 ± 2.7	117.9 ± 5.9	98.7 ± 4.3	86.5 ± 4.3	≤0.2
EDTA	11.6 ± 0.2	43.1 ± 2.1	≤0.2	≤0.2	≤0.2
ATP+Mn^{2+}	137.1 ± 0.8	140 ± 3.3	702.9	431.4 ± 1.2	≤0.2

In the cases of 7-DMATS and FtmPT1, H-L-Trp-Gly-OH was incubated with 0.6 μM protein. In the case of CdpNPT, FgaPT1, and FgaPT2, H-L-Trp-Gly-OH was incubated with 0.3 μM protein. All assays were incubated at 37°C for 45 min. The absolute conversion rates without additives were found to be 60.0%, 11.7%, 4.2%, and 2.9% for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively.

that with Ca^{2+}. These results prompted us to reinvestigate the peptidase activity of FgaPT2 in the presence of different metal ions as used for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1. In addition, experiments were carried out with Mn^{2+} at a final concentration from 2 mM to 50 mM. No peptidase activity could be observed for FgaPT2 under these conditions (Table 1).

Some peptidases or proteases are ATP-dependent (Chandu and Nandi, 2004). Addition of ATP to the enzyme assays increased the peptidase activities of 7-DMATS, FgaPT1, CdpNPT and FtmPT1 to 152, 145, 243, and 149%, respectively, in comparison to those without additives (Table 1). The peptidase activities of CdpNPT and FtmPT1 could be increased by a combination of ATP and Mn^{2+} to 703 and 431% of those without additives, respectively (Table 1).

In summary, the peptidase activities of the indole prenyltransferases FtmPT1 and CdpNPT were Mn^{2+}-dependent and could be enhanced by addition of ATP. For 7-DMATS and FgaPT1, enzymatic activity could be enhanced by addition of ATP, but not or only slightly enhanced by addition of metal ions such as Ca^{2+} or Mg^{2+}. However, based on their behavior in the presence of EDTA, it can be speculated that metal ions play also an important role for their peptidase activities.

Substrate Specificities and Kinetic Parameters of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 as Peptidases

To test and compare the substrate specificities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, 31 peptides were incubated with 0.6 μM monomeric 7-DMATS and FtmPT1 and with 0.3 μM dimeric FgaPT1 and CdpNPT, respectively. The assays for FtmPT1 and CdpNPT contained Mn^{2+} at a final concentration of 5 mM and the assays for 7-DMATS and FgaPT1 contained Ca^{2+} at a final concentration of 5 mM. The tested peptides included 17 linear dipeptides, 8 tryptophan-containing cyclic dipeptides, 3 tryptophan-containing linear tripeptides, 2 tryptophan-containing linear tetrapeptides, and 1 tryptophan-containing linear hexapeptide (Table 2). After incubation at 37°C for 45 min, the reaction mixtures were analyzed on HPLC and the product formation was determined by quantification of tryptophan. The identity of the enzymatic products was confirmed by LC-MS analysis (data not shown).

Our results showed that all linear peptides with a tryptophanyl moiety at N terminus, i.e., with a free amino group at tryptophanyl moiety, were accepted by all of the enzymes. All of linear dipeptides with a tryptophanyl moiety at C terminus, i.e., with a free carboxyl group at tryptophanyl moiety, were not accepted under the conditions tested in this study. In general, accepted linear dipeptides were significantly better substrates for all of the enzymes than peptides with three or more amino acids. An exception was the tetrapeptide amide H-L-Trp-L-Met-L-Asp-L-Phe-NH_2, which was better accepted by CdpNPT and FtmPT1 than some linear dipeptides (Table 2). High conversion rates were determined for H-L-Trp-L-Ala-OH and H-L-Trp-Gly-OH with a product formation of 179.1 and 151.6 mmol * mol protein^{-1} * s^{-1} for 7-DMATS, 45.6 and 65.6 mmol * mol protein^{-1} * s^{-1} for FgaPT1, 49.4 and 69.8 mmol * mol protein^{-1} * s^{-1} for FtmPT1, as well as 179.4 and 162.2 mmol * mol protein^{-1} * s^{-1} for CdpNPT (Table 2). By comparison of the product formation under the respective best condition, it is also obvious that 7-DMATS and CdpNPT showed comparable enzymatic activities with H-L-Trp-L-Ala-OH as the best substrate. FgaPT1 and FtmPT1 accepted H-L-Trp-Gly-OH and H-L-Trp-L-Phe-OH as the best substrate, respectively, and showed a relative activity of 36% and 46% to those of 7-DMATS and CdpNPT by using H-L-Trp-L-Ala-OH as substrate, respectively. Linear dipeptides lacking a tryptophanyl moiety such as H-Gly-L-Tyr-NH_2, H-L-Tyr-Gly-OH H-L-Phe-L-Ala-OH, and H-L-His-L-Leu-OH were accepted by none of the enzymes. Tryptophan-containing cyclic dipeptides were also not substrates of the tested enzymes. These results demonstrated clearly that the indole prenyltransferases function also as tryptophan aminopeptidases and accepted linear dipeptides as best substrates.

Table 2. Peptidase Activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1

Peptides	Product Formation (mmol product · mol protein^{-1} · s^{-1}) 7-DMATS	FgaPT1	CdpNPT	FtmPT1
H-L-Trp-L-Ala-OH	179.1	45.6	179.4	49.4
H-L-Ala –L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-Gly-OH	151.6	65.6	162.2	69.8
H-Gly-L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Lys-OH	112.5	28.8	37.4	40.4
H-L-Lys –L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Phe-OH	89.5	16.1	142.4	83.4
H-L-Trp-L-Asp-OH	58.0	14.6	38.5	49.0
H-L-Trp-L-Glu-OH	49.9	19.5	51.9	34.4
H-L-Glu-L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Pro-OH	1.6	≤0.5	≤0.5	≤0.5
H-L-Pro-L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Arg –L-Trp-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-Gly-L-Tyr-NH_2	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Tyr-Gly-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Phe-L-Ala-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-His-L-Leu-OH	≤0.5	≤0.5	≤0.5	≤0.5
H-L-Trp-L-Trp-L-Trp-OH	3.3	≤0.5	8.1	47.6
H-Gly-L-Trp-Gly-OH	3.9	≤0.5	20.3	≤0.5
H-L-Leu-L-Trp-L-Leu-OH	1.1	≤0.5	≤0.5	≤0.5
H-L-Trp-Gly-Gly-L-Tyr	5.2	≤0.5	≤0.5	37.2
H-L-Trp-L-Met-L-Asp-L-Phe-NH_2	2.9	≤0.5	85.3	48.2
H-L-Trp-L-Thr-L-Val-L-Pro-L-Thr-L-Ala-OH	3.0	≤0.5	14.1	≤0.5
cyclo-L-Trp-L-Pro	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-D-Trp-L-Pro	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-Gly-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Leu-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Phe-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Trp-L-Trp	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-L-Trp-L-Tyr	≤0.5	≤0.5	≤0.5	≤0.5
cyclo-D-Trp-L-Tyr	≤0.5	≤0.5	≤0.5	≤0.5

Assays of 7-DMATS and FgaPT1 contained $CaCl_2$ at a final concentration of 5 mM and assays of CdpNPT and FtmPT1 contained $MnCl_2$ at a final concentration of 5 mM.

Linear tri-, tetra-, and hexapeptides were also substrates of these enzymes with relatively lower conversion rates (with the exception of H-L-Trp-L-Met-L-Asp-L-Phe-NH_2, mentioned above), in comparison to linear dipeptides with a free amino group at the tryptophanyl moiety. Interestingly, H-Gly-L-Trp-Gly-OH, H-L-Leu-L-Trp-L-Leu-OH with a tryptophanyl moiety linked directly to two amino acids by two amide bonds were also accepted by the enzymes. This phenomenon was also observed for other aminopeptidases; e.g., leucine aminopeptidase LAP-A of potato also accepted tripeptides (Gu and Walling, 2000).

For a better understanding of this enzyme group, kinetic parameters of the peptidase activities were determined by using H-L-Trp-L-Gly-OH as substrate and compared with those of their prenyltransferase activities (Table 3). The peptidase reactions of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 followed Michaelis-Menten kinetics. Comparable K_m values for the peptidase activities were found to be 350 μM, 380 μM, 300 μM, and 420 μM for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. These values are significantly higher than those of the prenyltransferase activities in the presence of DMAPP; e.g., 137 μM for 7-DMATS by using L-tryptophan as substrate (Kremer et al., 2007), 6 μM for FgaPT1 by using fumigaclavine A as substrate (Unsöld and Li, 2006), 55 μM for FtmPT1 by using brevianamide F as substrate (Grundmann and Li, 2005), as well as 128 μM for CdpNPT by using cyclo-D-Trp-L-Tyr in the presence of DMAPP (W.-B. Yin and S.-M.L., unpublished data; Table 3). Higher turnover numbers (k_{cat}) were also found for the prenyltransferase activities than for the peptidase activities (Table 3). As consequence, much higher enzymatic rate constants (k_{cat} K_m^{-1}) were determined for the prenyltransferase activities than for the peptidase activities. The enzymatic rate constants for the peptidase activities were found to be comparable in the range of 0.14 to 0.53 $mM^{-1}s^{-1}$. In contrast, the values for the prenyltransferase activities varied from 1.68 $mM^{-1}s^{-1}$ for 7-DMATS to 133.33 $mM^{-1}s^{-1}$ for FgaPT1 (Table 3).

DISCUSSION

In this study, we demonstrated that the indole prenyltransferases 7-DMATS, FgaPT1, FtmPT1, and CdpNPT from *Aspergillus fumigatus* showed significant tryptophan aminopeptidase activities toward linear dipeptides. To the best of our knowledge, this is the first report on the catalytic promiscuity of prenyltransferases. The chemical transformations of both reactions differ not only in the involved functional groups, but also in the reaction mechanisms, i.e., forming of a C-C bond versus breaking of a C-N bond. Catalytic promiscuity in enzymes was observed with different proteins and plays a natural role in evolution and in the biosynthesis of natural products (Bornscheuer and Kazlauskas, 2004). The genes coding for the enzymes described in this study belong to different biosynthetic gene clusters of secondary metabolites and their function as prenyltransferases have been unequivocally proven by biochemical characterizations (Grundmann and Li, 2005; Kremer et al., 2007; Li and Unsöld, 2006; Ruan et al., 2008; Unsöld and Li, 2006; Yin et al., 2007). L-tryptophan is the substrate of the prenyltransfer reaction of 7-DMATS (Kremer et al., 2007) and tryptophan-containing cyclic dipeptides were accepted as substrates by FtmPT1 (Grundmann and Li, 2005) and CdpNPT (Ruan et al., 2008), while a somewhat complicated structure fumigaclavine A is the natural substrate for FgaPT1 (Unsöld and Li, 2006; Figure 1). A common feature of these substrates is the indole moiety in tryptophan or structures derived thereof. Interestingly, the second dimethylallyltryptophan synthase FgaPT2 involved in the biosynthesis of fumigaclavines (Unsöld and Li, 2005) showed no aminopeptidase activity under conditions described in this study. From our results, it can not be determined whether and which roles the aminopeptidase activities could play for the prenyltransfer reactions and/or for the biosynthesis of the respective secondary metabolites. No direct correlation could be found between prenyltransfer reactions and aminopeptidase activities. For

Table 3. K_m Values and Turnover Numbers of Prenyltransferase and Peptidase Activities of Recombinant Enzymes

	Prenyltransferase Activity			Peptidase Activity		
Enzyme	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]	K_m [mM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$mM^{-1}s^{-1}$]
7-DMATS	0.137	0.23	1.68	0.35	0.18	0.51
FgaPT1	0.006	0.80	133.33	0.38	0.09	0.24
CdpNPT	0.128	0.46	3.59	0.30	0.16	0.53
FtmPT1	0.055	5.57	101.27	0.42	0.06	0.14
FgaPT2	0.009	0.27	30.00	-	-	-

example, the natural substrates of the prenyltransfer reactions of 7-DMATS and FgaPT1 are L-tryptophan and fumigaclavine A, respectively, which lack a peptide structure as substrates for the peptidase activities. Tryptophan-containing cyclic dipeptides were no substrates for the peptidase activities of the prenyltransferases but they could be prenylated by CdpNPT and FtmPT1 in the presence of DMAPP (Grundmann and Li, 2005; Ruan et al., 2008). In case of 7-DMATS, however, it could be speculated that both peptidase and prenyltransferase activity could be involved in the biosynthesis of the same secondary metabolite. *7-dmats* belongs to a putative biosynthetic gene cluster containing a gene coding for a putative nonribosomal peptide synthetase (NRPS) EAL92291. The product of the gene cluster could be a derivative of a cyclic dipeptide containing 7-DMAT (Kremer et al., 2007). We have proposed that L-tryptophan could be at first converted to 7-DMAT catalyzed by the prenyltransferase 7-DMATS. 7-DMAT would then undergo the condensation with a second amino acid, which could not be predicted by the sequence analysis, to a prenylated (cyclic) dipeptide under the catalysis of EAL92291 (Kremer et al., 2007). Like the NRPS GliP in the biosynthesis of gliotoxin consisting of L-Phe and L-Ser, EAL92291 has a trimodular architecture (A1-T1-C1-A2-T2-C2-T3) (Balibar and Walsh, 2006). It has been proven by biochemical investigation that the A1 and A2 domains in GliP are responsible for the activation of L-Phe and L-Ser, respectively, which are then loaded onto the T1 and T2 domains of GliP, respectively. The linear enzyme-bound peptide L-Phe-L-Ser-T2 was formed under the catalysis of the condensation domain C1 (Balibar and Walsh, 2006). The roles of the second condensation domain C2 and the third thiolation domain T3 are unclear, although the mutational experiments suggested that they are involved in the same process (Balibar and Walsh, 2006). It was speculated that the subsequent reactions to gliotoxin, e.g., methylation or sulfur insertion, may occur while the linear dipeptide is still covalently bound to the NRPS GliP (Balibar and Walsh, 2006). The release of an intermediate in the biosynthesis could occur at some later step. This could also be the case for EAL92291. The peptidase activity of 7-DMATS could be involved in the release of the dipeptide from the NRPS EAL92291, because aminopeptidases often have esterase activity (Bornscheuer and Kazlauskas, 2004).

Furthermore, aminopeptidase activities of the indole prenyltransferases could be involved in peptide degradation and pathogenesis of their natural producer, i.e., *Aspergillus fumigatus*. Aminopeptidases perform essential cellular functions, including many normal and pathophysiological processes, and are widely distributed among bacteria, fungi, plants, and mammals (Gonzales and Robert-Baudouy, 1996; Lowther and Matthews, 2002; Taylor, 1993). Intracellular proteolytic degradation is important for the elimination of damaged proteins, the modulation of protein levels, and the maintenance of amino acid pools (Basten et al., 2001). In bacteria and fungi, aminopeptidases are involved in the utilization of exogenous peptides as nutrients after degradation with a variety of peptidases of different specificity (Gonzales and Robert-Baudouy, 1996). For the uptake of short chain peptides, *Aspergillus* species have not been reported to have many transport systems, and, therefore, the proteolytic enzymes should contain a range of different peptidases to enable the organism to degrade the various proteins to free amino acids (Garraway and Evans, 1984; Nampoothiri et al., 2005). Different aminopeptidases have been identified in *Aspergillus* species including leucine, lysine, phenylalanine, aspartate, and proline, but no tryptophan aminopeptidase (Basten et al., 2001, 2003, 2005; Chien et al., 2002; Nampoothiri et al., 2005; Watanabe et al., 2007). A literature search revealed only one known tryptophan aminopeptidase (EC 3.4.11.17) from the yeast *Trichosporon cutaneum* (Iwayama et al., 1983), although 2000 peptidase species have been characterized from different sources (Barrett and Rawlings, 2007). Therefore, it can be speculated that the degradation of tryptophan-containing peptides for primary and secondary metabolism is carried out by other aminopeptidases with broad substrate specificity or even by other enzyme groups, e.g., prenyltransferases reported in this study. The part of tryptophan released by indole prenyltransferases could be directly used without any transport process through different cell compartments for the biosynthesis of the respective tryptophan-containing natural products.

Aspergillus fumigatus produces during its mycelial growth different toxic metabolites such as gliotoxin and enzymes including different proteases, which could play a role in pathogenesis (Rementeria et al., 2005; Tomee and Kauffman, 2000). Although none of these factors has been shown to be involved in the pathogenesis of *Aspergillus fumigatus* in experimentally induced infections (Brakhage, 2005; Hohl and Feldmesser, 2007; Latgé, 2001), their involvement in the pathogenesis cannot be excluded. All data accumulated to date suggest that many factors are involved in the mycelial virulence of *Aspergillus fumigatus* (Hohl and Feldmesser, 2007; Latgé, 2001). It could be speculated that indole prenyltransferases are involved with their peptidase activities in the pathogenesis directly in the case of FgaPT1 and 7-DMATS or after induction by metal ions, e.g., Mn^{2+}, in the case of FtmPT1 and CdpNPT. As mentioned above, peptide degradation would supply amino acids, which are essential for the fungal growth and development.

A database search revealed no sequence similarity of the indole prenyltransferases to known proteases or peptidases.

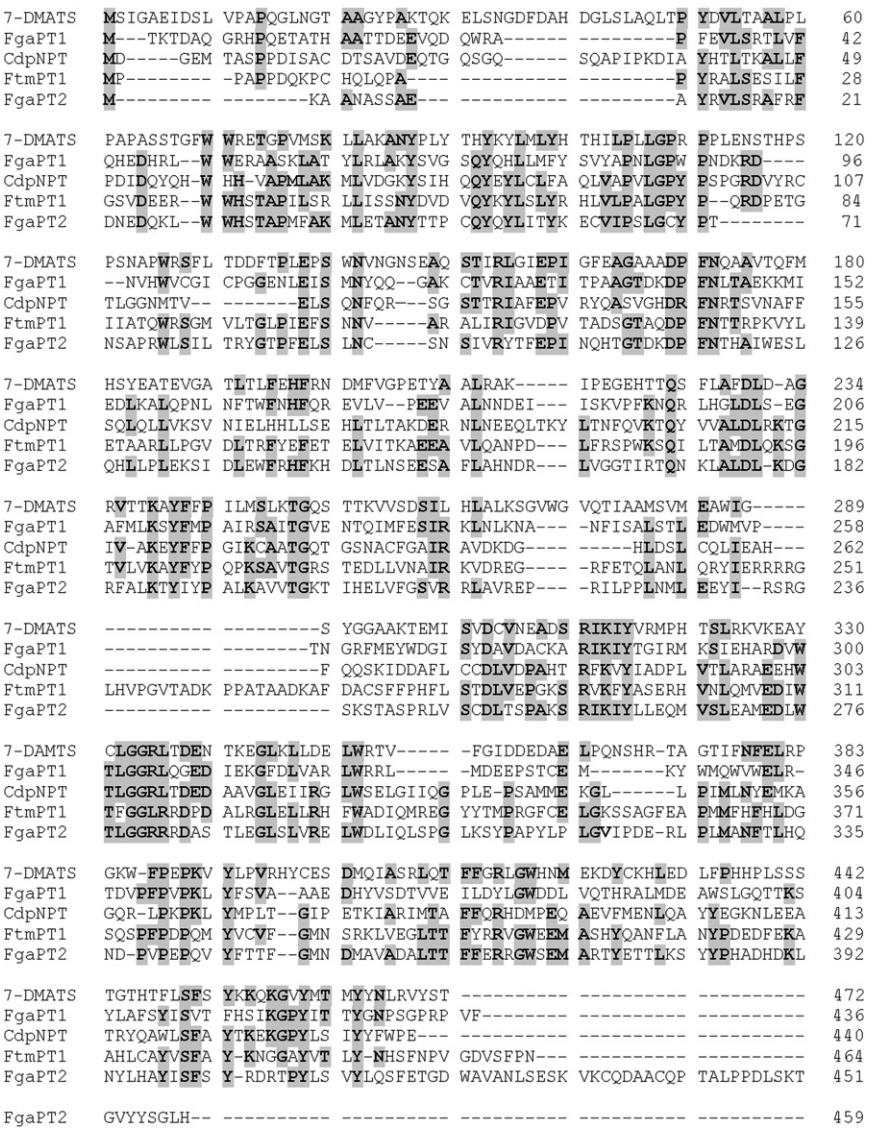

Figure 3. Alignments of Known Prenyltransferases from *Aspergillus fumigatus*

Sequence analysis of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 showed no conserved motifs for known metal binding sites, e.g., those found in a leucine aminopeptidase from *Aspergillus sojae* (Chien et al., 2002). Comparison of the sequences of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 with that of FgaPT2 (Figure 3) indicated no significant difference of motifs, which could be responsible for their different behavior regarding the peptidase activities.

The indole prenyltransferases 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 showed high substrate specificities for their aminopeptidase activities regarding the position of the tryptophanyl moiety in the peptide molecules. As shown in this study, tryptophan-containing linear dipeptides with an N-terminal tryptophan moiety, e.g., H-L-Trp-L-Ala-OH, H-L-Trp-Gly-OH, and H-L-Trp-L-Lys-OH, were well accepted as substrates for the aminopeptidase activities. The counter partners of these linear dipeptides, H-L-Ala–L-Trp-OH, H-Gly-L-Trp-OH, or H-L-Lys-L-Trp-OH, were not accepted (Table 2). This feature differed clearly from many known aminopeptidases from bacteria and fungi, which showed higher substrate flexibility (Chien et al., 2002; Gonzales and Robert-Baudouy, 1996; Gu and Walling, 2000; Herrera-Camacho et al., 2000; Monod et al., 2005). Aminopeptidases belong to the large group of metallopeptidases and their reactions are dependent on divalent metal ions such as Zn^{2+}, Mg^{2+}, and Mn^{2+} (Gonzales and Robert-Baudouy, 1996; Lowther and Matthews, 2002; Taylor, 1993). The most investigated aminopeptidases are leucine, lysine, cysteine, serine, and methionine aminopeptidases (Gonzales and Robert-Baudouy, 1996; Lowther and Matthews, 2002; Nampoothiri et al., 2005). The tryptophan aminopeptidase from *Trichosporon cutaneum* mentioned above (Iwayama et al., 1983) showed broad substrate specificity toward linear dipeptides and accepted also different simple amino acid amides as substrates (Iwayama et al., 1983). Mn^{2+} was essential for the enzymatic reaction of the aminopeptidase from *Trichosporon cutaneum* (Iwayama et al., 1983), which is consistent with other known aminopeptidases.

Correspondingly, the aminopeptidase activities of 7-DMATS, FgaPT1, CdpNPT, and FtmPT1 are dependent on the presence of metal ions. Strong or complete inhibition of the peptidase activities was observed after addition of EDTA to the reaction mixtures (Table 1), indicating the importance of metal ions, which were removed by EDTA. Direct support for this speculation was provided by addition of Mn^{2+} to the reaction mixtures of FtmPT1 and CdpNPT. The peptidase activities could be increased up to 4- and 6-fold for FtmPT1 and CdpNPT, respectively (Table 1). These data proved that Mn^{2+} ions are involved in the peptidase reaction of the indole prenyltransferases, at least for those of FtmPT1 and CdpNPT. Addition of Mn^{2+} to the reaction mixtures of 7-DMATS and FgaPT1 reduced the peptidase activities to 84% and 70% of those without additives, respectively. Ca^{2+} and Mg^{2+} enhance the peptidase activities of FgaPT1 and FtmPT1 slightly. It is plausible that 7-DMATS and FgaPT1 bind metal ions more tightly than FtmPT1 and CdpNPT and contain enough metal ions for their peptidase activities, even after protein purification on Ni-NTA agarose. These metal ions could be partly or completely removed by addition of EDTA, resulting in decreasing of peptidase activities. Supports for this explanation were provided by the fact that 7-DMATS and FgaPT1 showed higher peptidase activities without additives than FtmPT1 and CdpNPT. The conversion rates of H-L-Trp-Gly-OH without

additives were found to be 60.0%, 11.7%, 4.2%, and 2.9% for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. Considering the different enzyme amounts in the assays (0.6 μM 7-DMATS and FtmPT1, 0.3 μM FgaPT1 and CdpNPT), relative peptidase activities of 100%, 39.0%, 14.0%, and 4.8% could be calculated from these data for 7-DMATS, FgaPT1, CdpNPT, and FtmPT1, respectively. After addition of Mn^{2+} to the reaction mixtures, the enzyme activities of FtmPT1 and CdpNPT could be reached to 422% and 591% of those without additives, respectively.

The dependence of the amiopeptidase activities of indole prenyltransferases on metal ions is sharply in contrast to their prenyltransferase activities. No divalent metal ions are essential for the prenyltransferase reactions, although Ca^{2+} enhances the enzyme activities. No or nearly no loss of the prenyltransferase activities was observed after addition of EDTA to the reaction mixtures of all of the purified and characterized prenyltransferases (Grundmann and Li, 2005; Kremer et al., 2007; Li and Unsöld, 2006; Unsöld and Li, 2006; Yin et al., 2007). Similar phenomenon was also observed with TdiB, an indole prenyltransferase from *Aspergillus nidulans* (Schneider et al., 2008). The requirements of the prenyltransfer and hydrolysing reactions on metal ions could indicate different active sites of the enzymes for both reactions. However, it was also reported that catalytic promiscuity could be created by binding of different metal ions at the same active site (Bornscheuer and Kazlauskas, 2004). Site-directed mutagenesis or protein structure would provide information on the active sites of the prenyltransferase and peptidase activities of the indole prenyltransferases from fungi.

We have speculated that the indole prenyltransferases from fungi have evolved directly from a common ancestor (Kremer et al., 2007; Yin et al., 2007). Our results reported here provide further support for this hypothesis. Divergent evolution is a natural process that creates different species from a common ancestor, which is also applicable for creation of enzymes with new catalytic activities. New enzymatic activities arise by gene duplication, followed by evolution of new activity for the copy (Bornscheuer and Kazlauskas, 2004; Gerlt and Babbitt, 2001). It could be speculated that an earlier protein carried only one enzymatic activity and the second one was created after gene duplication. It cannot be proposed, however, which activity was the first enzymatic activity of this enzyme group. From the much higher catalytic efficiencies of the prenyltransferase activities in comparison to those of peptidase activities (Table 3), on the other hand, it could be postulated that the prenyltransferase activities were present before the peptidase activities. During the evolution, some enzymes adapted the second catalytic activity, namely, the peptidase activity (e.g., 7-DMATS, FgaPT1, FtmPT1 and CdpNPT described in this study). Others have still only prenyltransferase activities (e.g., FgaPT2). It seems that specifications for the prenyltransferase activities have also taken place during the evolution. The substrate specificities and enzymatic rate constants of the prenyltransfer reactions catalyzed by these enzymes differed clearly from each other. On the other hand, these parameters are similar for their peptidase activities. But the peptidase activities of some enzymes, e.g., CdpNPT and FtmPT1, can be induced at least in vitro by metal ions such as Mn^{2+}. It would be interesting to investigate the aminopeptidase activities of other prenyltransferases from fungi, plants, or bacteria.

Peptidases are widely used in research and diagnostics and in the leather, food, and pharmaceutical industries (Rao et al., 1998), e.g., for post-hydrolysis of protein hydrolysates. Protein hydrolysates are acidic, alkaline, or enzymatic hydrolysis products of proteins; contain short-chain peptides and free amino acids; and are used as constituents of dietetic and health products, clinical nutrition supplements, or as flavoring agents (Clemente, 2000; Rao et al., 1998). The bitter taste of protein hydrolysates is a major barrier for their use in food and health care products (Clemente, 2000; Rao et al., 1998). The intensity of the bitterness is proportional to the number of hydrophobic amino acids in the hydrolysates (Clemente, 2000; Ishibashi et al., 1988; Rao et al., 1998). Exopeptidases such as aminopeptidases can reduce the amount of peptides with undesirable tastes through the removal of a single hydrophobic amino acid, or pairs of them, from the terminal ends and are therefore used in the posthydrolysis processes in the production of protein hydrolysates (Clemente, 2000; Rao et al., 1998). Tryptophan is the most hydrophobic of amino acid side chains (Nozaki and Tanford, 1971) and tryptophan-containing peptides therefore also contribute to the bitterness of protein hydrolysates (Lalasidis, 1978). For this reason, tryptophan aminopeptidase could find application for debittering of protein hydrolysates by removal of N-terminal tryptophan moiety. In analogy to many commercial aminopeptidases, a further possible application of the enzymes described in this study could be for determination of tryptophan-containing peptides, especially dipeptides.

SIGNIFICANCE

The results described in this study showed that four of five identified indole prenyltransferases carried also tryptophan aminopeptidase activities. To the best of our knowledge, this is the first report on the catalytic promiscuity of prenyltransferases. Similar substrate specificity was observed for the peptidase activities, while different indole derivatives were substrates of the prenyltransfer reactions. The aminopeptidase activities of all of the four enzymes could be inhibited by addition of EDTA. In addition, the aminopeptidase activities of FtmPT1 and CdpNPT were strictly dependent on the presence of Mn^{2+} ions and were strongly enhanced by addition of Mn^{2+} to the reaction mixtures. This is in contrast to the prenyltransfer reactions catalyzed by these enzymes, which are independent of the presences of metal ions. The results described in this study provide further supports for the hypothesis that the indole prenyltransferases from fungi have evolved directly from a common ancestor.

EXPERIMENTAL PROCEDURES

Chemicals

The synthesis of brevianamide F has been reported previously (Grundmann and Li, 2005). The other peptides were purchased from Bachem.

Protein Overproduction and Purification

Overproduction and purification of His_6-7-DMATS, His_6-FgaPT1, His_6-CdpNPT, His_6-FtmPT1, and His_8-FgaPT2 were described previously (Grundmann and Li, 2005; Kremer et al., 2007; Steffan et al., 2007; Unsöld and Li, 2006; Yin et al., 2007).

Determination of Molecular Weight of Native Enzymes

The molecular weights of native His_6-FgaPT1 and His_6-FtmPT1 were reported previously (Grundmann and Li, 2005; Unsöld and Li, 2006).

The molecular weights of native His_6-7-DMATS and His_6-CdpNPT were determined by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Health Care) which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2000 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (13.7 kDa), and aprotinin (6.5 kDa) (GE Healthcare). The proteins were eluted with 50 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The molecular weights of native His_6-7-DMATS and His_6-CdpNPT were determined as 65 kDa and 104 kDa, respectively. This proved that His_6-7-DMATS acts as a monomer and His_6-CdpNPT acts as a homodimer.

Assay for Peptidase Activity

For quantitative determination of the enzyme activity of 7-DMATS and FtmPT1, the reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.5), 2 mM aromatic substrates, 1.5% (v/v) glycerol, 0.6 μM purified recombinant proteins, and 5 mM $CaCl_2$ in the case of 7-DMATS (5 mM $MnCl_2$ in the case of FtmPT1). For quantitative determination of the enzyme activity of FgaPT1 and CdpNPT, the reaction mixture (100 μl) contained 50 mM Tris-HCl (pH 7.5), 2 mM aromatic substrates, 1.5% (v/v) glycerol, 0.3 μM purified recombinant proteins, and 5 mM $CaCl_2$ in the case of FgaPT1 (5 mM $MnCl_2$ in the case of CdpNPT). After incubation for 45 min at 37°C, the reaction was stopped with 100 μl methanol. The protein was removed by centrifugation at 13,000 × g for 10 min. The enzymatic products were analyzed by HPLC under conditions described below. For quantitative measurement of the enzyme activity, duplicate values were determined routinely.

HPLC Analysis

The enzymatic products of the incubation mixtures were analyzed by HPLC on an Agilent HPLC Series 1100 by using an Eclipse XDB-C18 column (4.6 × 150 mm, 5 μm [Agilent]) at a flow rate of 1 ml min^{-1}. For separation, a linear gradient of 30%–70% (v/v) methanol containing 0.5% (v/v) aqueous trifluoroacetic acid (TFA) in 15 min was used. The column was then washed with 100% methanol containing 0.5% (v/v) TFA for 5 min and equilibrated with 30% (v/v) methanol containing 0.5% (v/v) TFA for 5 min. Detection was carried out by a Photo Diode Array detector.

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A tyrosine O-prenyltransferase catalyses the first pathway-specific step in the biosynthesis of sirodesmin PL

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Running title: Tyrosine O-prenyltransferase from *Leptosphaeria maculans*

Abstract

A putative prenyltransferase gene *sirD* was identified in the biosynthetic gene cluster of the phytotoxin sirodesmin PL in *Leptosphaeria maculans*. The gene product comprises 449 amino acids with a molecular mass of 51 kDa. In this study, the coding region of *sirD* was amplified by PCR from cDNA, cloned into pQE70 and overexpressed in *Escherichia coli*. The overproduced protein was purified to apparent homogeneity and characterised biochemically. The dimeric recombinant SirD was found to catalyse the O-prenylation of L-tyrosine in the presence of dimethylallyl diphosphate, which was proven unequivocally by isolation and structural elucidation of the enzymatic product. Therefore, SirD catalyses the first pathway-specific step in the biosynthesis of sirodesmin PL. K_M values for L-tyrosine and dimethylallyl diphosphate were determined as 0.13 and 0.17 mM, respectively. Interestingly, SirD shares significant sequence similarity with indole prenyltransferases, which catalyse prenyl transfer reactions onto different positions of indole rings. In contrast to indole prenyltransferases, which accepted indole derivatives, but not tyrosine or structures derived thereof as substrates, SirD prenylated also L-tryptophan resulting in formation of 7-dimethylallyltryptophan. A K_M value of 0.23 mM was determined for L-tryptophan. Turnover numbers were calculated for L-tyrosine and L-tryptophan at 1.0 and 0.06 S^{-1}, respectively.

Key words: *Leptosphaeria maculans*, sirodesmin biosynthesis, tyrosine O-prenyltransferase

INTRODUCTION

Filamentous fungi produce diverse secondary metabolites exhibiting a large variety of biological and pharmacological activities (Hoffmeister & Keller 2007; Nielsen & Smedsgaard 2003). Some of these compounds are mycotoxins like epipolythiodioxopiperazines (ETP) (Gardiner *et al.,* 2005). ETPs are characterised by the presence of a sulphurbridged diketopiperazine ring system derived from two amino acids (Gardiner & Howlett 2005). This disulphide bridge allows ETP to cross-link proteins *via* cysteine residues and to generate reactive oxygen species through redox cycling (Mullbacher *et al.,* 1986). Two prominent members are gliotoxin derived from L-phenylalanine and L-serine and sirodesmin PL from L-tyrosine and L-serine (Gardiner *et al.,* 2005). Gliotoxin was identified in different fungal strains including the opportunistic human pathogen *Aspergillus fumigatus* (*A. fumigatus*) (Glister & Williams 1944). It was speculated that this mycotoxin could be involved in the pathogenesis of *A. fumigatus* (Kwon-Chung & Sugui 2009). Sirodesmin PL (Fig. 1) was first identified in the asexual stage of the ascomycetous fungus *Leptosphaeria maculans* (*L. maculans*), *i.e. Phoma lingam* (Ferezou *et al.,* 1977). *L. maculans* causes blackleg of canola (*Brassica napus*), the most damaging disease of this crop (Howlett *et al.,* 2001). Sirodesmin PL is reported to contribute to virulence of *L. maculans* during growth in stem of *B. napus* (Elliott *et al.,* 2007). On the other hand, sirodesmin PL exhibited also activity against gram-positive bacteria (Boudart 1989).

Feeding experiments in *Phoma lingam* showed the incorporation of ^{13}C- and ^{14}C-labelled precursors such as acetate, L-tyrosine, L-serine into the structures of sirodesmin PL and the O-prenylated cyclic dipeptide phomamide (Fig. 1) (Bu´Lock & Clough 1992; Ferezou *et al.,* 1980). Incorporation of ^{14}C-labelled cyclo-L-Tyr-L-Ser in sirodesmin PL from the same experiment (Ferezou *et al.,* 1980) suggested its involvement as an intermediate in the biosynthesis of sirodesmin PL (Ferezou *et al.,* 1980). It was therefore proposed that the cyclic

dipeptide was firstly formed by condensation of L-tyrosine and L-serine and then converted to phomamide by prenylation (Fig. 1) (Ferezou *et al.*, 1980). However, no report on an enzymatic conversion was found in the literature.

In a previous study, a putative gene cluster consisting of 18 genes was identified for the biosynthesis of sirodesmin PL in *L. maculans* (Gardiner *et al.*, 2004). Inactivation of the non-ribosomal peptide synthetase gene *sirP* from this cluster resulted in abolishment of sirodesmin production, proving its involvement in the biosynthesis (Gardiner *et al.*, 2004). From this cluster, a putative prenyltransferase gene *sirD* was identified and proposed to be responsible either for the O-prenylation of L-tyrosine resulting in formation of 4-O-dimethylallyl-L-tyrosine or of the cyclic dipeptide cyclo-L-Tyr-L-Ser resulting in formation of phomamide (Fox & Howlett 2008; Gardiner *et al.*, 2004). No experimental data were available on the function of SirD. Interestingly, the proposed tyrosine or cyclo-L-Tyr-L-Ser O-prenyltransferase SirD shares significant sequence similarity with 7-DMATS from *A. fumigatus*. 7-DMATS catalyses the C-prenylation of L-tryptophan at position C7 of the indole moiety, but did not accept L-tyrosine or a derivative thereof as substrate (Kremer *et al.*, 2007). To prove the function of SirD and its role in the biosynthesis of sirodesmin PL, we cloned and overexpressed *sirD* in *E. coli* and characterised the overproduced protein biochemically.

METHODS

Chemicals: Trisammonium salt of dimethylallyl diphosphate (DMAPP) was synthesised in analogy to the synthesis of trisammonium geranyl diphosphate reported by Woodside (1988). The amino acids and derivatives were purchased from Sigma-Aldrich (Munich, Germany) and Bachem (Bubendorf, Switzerland).

Computer-assisted sequence analysis: FGENESH (Softberry, Inc.,

www.softberry.com/berry.phtml) and the DNASIS software package (version 2.1: Hitachi Software Engineering, San Bruno, CA) were used for exon/intron prediction and sequence analysis, respectively. Sequence similarities were obtained by alignments of amino acid sequences using the BLAST program “BLAST 2 SEQUENCES”.

Bacterial strains, plasmids and cultural conditions for *E. coli*: pGEM-T easy and pQE70 were obtained from Promega (Mannheim, Germany) and Qiagen (Hilden, Germany), respectively. *Escherichia coli* XL1 Blue MRF´ (Stratagene, Waldbronn, Germany) was used for cloning and expression experiments and grown in liquid or on solid Luria-Bertani medium with 1.5 % (w/v) agar at 37 °C (Sambrook & Russell 2001). Carbenicillin (50 µg ml^{-1}) was used for selection of recombinant *E. coli* strains.

Fungal culture and RNA isolation: The isolate ICBN (M1) of *Leptosphaeria maculans* was kindly provided by Dr. Howlett (The University of Melbourne, Australia). The fungus was grown on 100 ml potatoe dextrose media without agitation. After separating the mycelia from the media, RNA was isolated using a HiBind matrix (Omega Bio-Tek, Norcross, USA) according to the manufacturer´s protocol. cDNA synthesis was carried out by using a cDNA synthesis kit (Roche Diagnostics GmbH, Mannheim, Germany).

DNA isolation, PCR amplification and cloning: Standard procedures for DNA isolation and manipulation were performed as described (Sambrook & Russell 2001). PCR amplification was carried out on an iCycler from BioRad (Munich, Germany).

A PCR fragment of 1354 bp containing the entire coding sequence of *sirD* was amplified using Expand High Fidelity Kit (Roche Diagnostics GmbH, Mannheim, Germany) from the obtained cDNA of *L. maculans* by using the primers sirD-1 (5´ **GC**ATGCAGACAGCTCGTCTCTTCC’-3´) at the 5´-end and sirD-2 (5´-ATA**GATC**TCTGTCTGTAGCGATTTGGA -3´) at the 3´-end of the gene. Bold letters represent mutations inserted in comparison to the original genomic sequence to give the

underlined restriction sites SphI located at the start codon in sirD-1 and BglII located at the predicted stop codon in sirD-2. The PCR fragment was cloned into pGEM-T easy resulting in plasmid pAK1, which was subsequently sequenced (Eurofins MWG Operon, Ebersberg, Germany) to confirm the sequence. To create the expression vector pAK2, pAK1 was digested with SphI and BglII and the resulted SphI - BglII fragment of 1341 bp was ligated into pQE70, which had been digested with the same enzymes, previously.

Overproduction and purification of His_6-SirD: For gene expression, *E. coli* XL1 Blue MRF´ cells harbouring the plasmid pAK2 were cultivated in 300 ml Erlenmeyer flasks containing 100 ml liquid Luria-Bertani medium supplemented with carbenicillin (50 µg ml^{-1}) and grown at 37 °C to an A_{600} of 0.6. For induction, isopropyl thiogalactoside (IPTG) was added to a final concentration of 0.5 mM and the cells were cultivated for further 16 h at 37 °C before harvest. The bacterial cultures were centrifuged and the pellets were resuspended in lysis buffer (10 mM imidazole, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) at 2-5 ml per gram wet weight. After addition of 1 mg ml^{-1} lysozyme and incubation on ice for 30 min, the cells were sonicated 6 times for 10 seconds each at 200 W. To separate the cellular debris from the soluble proteins, the lysate was centrifuged at 14,000 x *g* for 30 min at 4°C. One-step purification of the recombinant His_6-tagged fusion protein by affinity chromatography with Ni-NTA agarose resin (Qiagen, Hilden, Germany) was carried out according to the manufacturer's instructions. The protein was eluted with 250 mM imidazole in 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0. In order to change the buffer, the protein fraction was passed through a NAP-5 column (GE Health Care, Freiburg, Germany), which had been equilibrated with 50 mM Tris-HCl, 15 % (v/v) of glycerol, pH 7.5, previously. His_6-SirD was eluted with the same buffer and stored frozen at - 80 °C for enzyme assays.

Protein analysis and determination of molecular mass of active His_6-SirD: Protein monomers were analysed by SDS-PAGE according to the method of Laemmli (Laemmli 1970)

and stained with Coomassie Brilliant Blue G-250.

The molecular mass of the recombinant active His_6-SirD was determined by gel filtration on a HiLoad 16/60 Superdex 200 column (GE Health Care, Freiburg, Germany), which had been equilibrated with 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The column was calibrated with dextran blue 2000 (2000 kDa), conalbumin (75 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa) and ribonuclease A (13.7 kDa) (GE Health Care, Freiburg, Germany). The protein was eluted with 20 mM Tris-HCl buffer (pH 7.5) containing 150 mM NaCl. The molecular mass of the recombinant active His_6-SirD was determined as 110 kDa. This proved that SirD acts as a dimer.

Prenyltransferase assay for SirD: All of the enzyme assays contained 50 mM Tris-HCl, pH 7.5, 1.5 % (v/v) of glycerol and 5 mM $CaCl_2$. The reaction mixtures were incubated at 37 °C and the reactions were terminated by addition of 100 µl methanol per 100 µl reaction mixtures. After removal of the protein by centrifugation (14,000 x *g*, 10 min, 4 °C), the enzymatic products were analysed on a HPLC system described below. Two independent assays were carried out routinely. The assays for determination of the kinetic parameters (100 µl) of L-tyrosine contained 1 mM DMAPP, 48 nM of SirD and L-tyrosine at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5 or 1.0 mM. The incubation time was 30 min. For determination of the kinetic parameters of DMAPP, 48 nM of SirD, L-tyrosine at 1 mM and DMAPP at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5 or 1.0 mM were used. For determination of the kinetic parameters of L-tryptophan, 48 nM of SirD, 1 mM DMAPP and L-tryptophan at a final concentration of 0, 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.5 or 1.0 mM were used.

Preparative synthesis of prenylation products for structural elucidation: SirD assay with L-tyrosine for isolation of the enzymatic product (5 mL) contained DMAPP (1 mM), L-tyrosine (1 mM), $CaCl_2$ (5 mM), Tris-HCl (50 mM, pH 7.5), glycerol (3 %, v/v) and SirD

(0.19 µM). SirD assay with L-tryptophan for isolation of the enzymatic product (10 mL) contained DMAPP (1 mM), L-tryptophan (1 mM), $CaCl_2$ (5 mM), Tris-HCl (50 mM, pH 7.5), glycerol (3 %, v/v) and SirD (0.19 µM). The reaction mixtures were incubated in glass vials at 37 °C for 16 h and were stopped with one volume of methanol. Conversion rates of 40 and 10 % could be detected for L-tyrosine and L-tryptophan, respectively. The reaction mixtures were concentrated on a rotation evaporator at 35 °C to a volume of 750 µl and centrifuged at 14,000 x *g* for 10 min before injection into HPLC for isolation of the enzymatic products. The fractions containing the prenylated products were collected and the solvents were evaporated. 0.3 mg of O-dimethylallyl-L-tyrosine and 0.2 mg of 7-DMAT were obtained and analysed by ^{1}HNMR spectroscopy.

HPLC conditions for analysis of incubation mixtures with SirD and isolation of the prenylation products: The prenyltransferase assays of SirD were analysed by HPLC on an Agilent series 1200 by using a LiChrospher RP 18-5 column (125 x 4 mm, 5 µm, Agilent) at a flow rate of 1 ml min^{-1}. Double distilled water (solvent A) and methanol (solvent B), both containing 0.5 % trifluoroacetic acid (TFA), were used as solvents. For analysis of the prenylation products, a linear gradient of 50-100 % (v/v) solvent B in 15 min was used. The column was then washed with 100 % solvent B for 5 min and equilibrated with 50 % (v/v) solvent A for 5 min. Detection was carried out by a Photo Diode Array detector and illustrated at 277 nm in this paper.

For structural elucidation, the prenylation products were isolated by using the same HPLC equipment as well as the same linear gradient. Double distilled water (solvent A) and methanol (solvent B) without TFA were used as solvents for this purpose.

Spectroscopic analysis: ^{1}HNMR spectra were measured on an ECA-500 spectrometer (JEOL) using DMSO as solvent. The positive electrospray ionisation (ESI) mass spectrometry was carried out with an AutoSpec instrument (Micromass Co. UK Ltd).

^{1}HNMR data of 4-O-dimethylallyl-L-tyrosine: δppm (DMSO-d_6): 7.12 (d, 8.6, H-2,6), 6.81 (d, 8.6, H-3,5), 5.38 (t sept, 6.8, 0.9, H-2´), 4.45 (d, 6.8, H-1´), 3.2 (t, 8.7, H-8), 3.02 (dd, 14.3, 5.0, H-7), 2.80 (dd, 14.3, 7.4, H-7), 1.66 (d, 0.9, 3H-4), 1.70 (d, 0.9, 3H-5).

ESI-MS data of 4-O-dimethylallyl-L-tyrosine: m/z (relative intensity): 272 ($[M+Na]^+$, 100), 250 ($[M+H]^+$, 88), 186 (15), 142 (25).

^{1}HNMR data of 7-dimethylallyltryptophan: The obtained data are identical to those reported previously (Kremer *et al.,* 2007).

RESULTS

Sequence analysis of the putative prenyltransferase gene *sirD.*

The genomic sequence of the putative prenyltransferase gene *sirD* spans bp 58737-60134 of AY553235.1, the nucleotide sequence of the genomic DNA of sirodesmin cluster at GenBank. Analysis by FGENESH revealed that *sirD* probably consists of two exons with 1217 and 133 bp, respectively, interrupted by an intron of 51 bp situated near the 3´ end of the gene. The deduced gene product SirD comprises 449 amino acids and has a calculated molecular mass of 51 kDa. SirD showed significant sequence similarity to known indole prenyltransferases, which catalyse prenyl transfer reactions onto different positions of the indole nucleus of tryptophan or derivatives thereof (Li 2009b; Steffan *et al.,* 2009). For example, by using the BLAST 2 SEQUENCES program, SirD shares a sequence similarity of 34 % on the amino acid level with 7-DMATS and 31 % with FgaPT2, both from *A. fumigatus*. 7-DMATS and FgaPT2 catalyse the prenylation of L-tryptophan at position C7 and C4 of the indole nucleus, respectively (Kremer *et al.,* 2007; Unsöld & Li 2005). Furthermore, SirD shows lower, but clear sequence similarities to other indole prenyltransferases, *e.g.* CdpNPT, FtmPT1 and FtmPT2 from *A. fumigatus* (Grundmann & Li 2005; Ruan *et al.,* 2008; Yin *et al.,* 2007). Most of the indole prenyltransferases mentioned above showed broad substrate specificity and

accepted simple indole derivatives and tryptophan-containing cyclic dipeptides as prenylation substrates. However, none of the known indole prenyltransferases prenylated L-tyrosine or a structure derived thereof (Li 2009b; Steffan *et al.*, 2009).

Cloning and expression of *sirD* as well as purification of His_6-SirD.

The coding region of *sirD* was amplified by PCR from mRNA of *L. maculans* M1 and cloned into the expression vector pQE70, resulting in the expression plasmid pAK2 (see METHODS). Gene expression with pAK2 in *E. coli* was induced by 0.5 mM IPTG at 37 °C for 16 h. Protein overproduction was clearly observed after induction (Fig. 2). SirD was subsequently purified on nickel-NTA agarose to apparent homogeneity as judged by SDS-PAGE and a protein yield of 2 mg of purified His_6-tagged SirD per litre of culture was obtained. The observed molecular mass was 50 kDa and corresponded well to the calculated mass of 52 kDa for His_6-SirD (Fig. 2).

Enzymatic activity of SirD and identification of the enzymatic product

To prove the enzymatic activity, SirD was incubated with L-tyrosine or cyclo-L-Tyr-L-Ser in the presence of dimethylallyl diphosphate (DMAPP) and 5 mM of $CaCl_2$. HPLC analysis of the incubation mixtures was used to monitor the formation of an enzymatic product. As shown in Fig. 3, product formation was detected only in the incubation mixture of L-tyrosine with DMAPP (Fig. 3A), but not in the incubation mixture of cyclo-L-Tyr-L-Ser with DMAPP (Fig. 3C). The product peak with a retention time of 6.7 min was absent in a control assay of L-tyrosine and DMAPP with heat-inactivated SirD by boiling of the enzyme solution for 20 min (Fig. 3B), demonstrating its dependence on the presence of active enzyme. Product formation was also strictly dependent on the presence of DMAPP (data not shown). Product

formation with L-tyrosine showed a linear dependence on the amount of protein up to 2 µg per 100 µl assay and on the incubation time up to 50 min.

For structural elucidation, incubation on a 5 ml scale with higher protein concentration (190 instead of 49 nM in the standard assay) was carried out (See METHODS). A conversion rate of 39.5 % could be achieved after incubation for 16 h. The enzymatic product was initially isolated on HPLC using water and methanol containing 0.5 % TFA as solvents and subjected to ^{1}HNMR analysis after evaporation of the solvents. Under this HPLC condition, the enzymatic product was well separated from the substrate, *i.e.* L-tyrosine. Surprisingly, the ^{1}HNMR spectrum of the isolated product in DMSO-d_6 indicated the presence of two substances. The dominant compound had identical data as those of tyrosine and the minor one showed signals for a dimethylallyl moiety (data not shown). HPLC analysis of the sample after NMR analysis confirmed that the dominant compound is indeed tyrosine and the minor product has the same retention time as the enzymatic product (data not shown). This observation could be interpreted as a result of hydrolysis of the enzymatic product to tyrosine during or after the evaporation of solvents of the collected fractions. These results indicated that the prenylation had very likely taken place at the phenolic hydroxyl group of tyrosine rather than at a carbon atom and the O-prenylated tyrosine was hydrolysed to tyrosine in the presence of TFA. To prove this hypothesis, the enzymatic product was isolated again on HPLC using water and methanol without TFA as solvents. ^{1}HNMR analysis of the isolated product revealed clearly the presence of only one compound bearing a dimethylallyl moiety, which is characterised by the signals at 5.38 (t sept, 6.8 Hz, 0.9 Hz, H-2'), 4.45 (d, 6.8 Hz, H-1'), 1.70 (d, 0.9 Hz, 3H-5') and 1.66 (d, 0.9 Hz, 3H-4') ppm, respectively. The signal of H-1' of the dimethylallyl moiety at 4.45 ppm indicated that the prenyl moiety was attached to a hetero atom (Chu *et al.,* 1993; Yin *et al.,* 2007) rather than a carbon atom. In case of a C-C connection, the signal of H-1' would be found in the range of 3.4 - 3.8 ppm (Grundmann & Li 2005; Kremer & Li 2008; Steffan *et al.,* 2007; Unsöld & Li 2005). In the spectrum of the

isolated product, the signals for the four aromatic protons as an AA´BB´ system were also observed at 7.12 and 6.81 ppm. This proved unequivocally that the enzymatic product of SirD is the expected 4-O-dimethylallyl-L-tyrosine (Fig. 3), which was also confirmed by detection of $[M+1]^+$ at m/z 250 in ESI-MS of the isolated compound.

Substrate specificity of SirD

SirD accepted only DMAPP, but not geranyl diphosphate as prenyl donor. Incubation of SirD with geranyl diphosphate did not result in formation of any enzymatic product (data not shown). To test the stereoselectivity of SirD towards its aromatic substrate, D-tyrosine was incubated instead of L-tyrosine. A relative activity of 12.3 % in comparison to that of L-tyrosine was detected for D-tyrosine (Table 1), being comparable to other prenyltransferases. FgaPT2 and 7-DMATS from *A. fumigatus* showed a relative activity of 9.7 and 11.8 % of that of L-tryptophan for D-tryptophan, respectively (Kremer & Li 2008; Steffan *et al.*, 2007). Incubation of SirD with L-phenylalanine, 4-hydroxybenzoic acid and *para*-coumaric acid in the presence of DMAPP did not result in formation of any enzymatic product (Table 1), demonstrating the absolute importance of the hydroxyl group at position C4 of the aromatic nucleus and of the amino acid structure at the side chain. An additional hydroxyl group at position C3 of the aromatic ring of tyrosine as in the case of L-3,4-dihydroxyphenylalanine resulted in a reduced, but still significant enzymatic activity of 16.5 % in comparison to that of L-tyrosine. It could be expected that the prenylation has taken place at the 4-hydroxyl group. However, the structure of the product with L-3,4-dihydroxyphenylalanine was not determined in this study. Neither cyclo-L-Tyr-L-Ser nor other tyrosine-containing cyclic dipeptides such as cyclo-L-Tyr-L-Pro, cyclo-L-Tyr-L-Trp or cyclo-L-Tyr-Gly were substrates for SirD under conditions tested in this study (Table 1).

As mentioned above, SirD shares significant sequence similarity with the

7-dimethylallyltryptophan synthase 7-DMATS from *A. fumigatus*. Therefore, we wondered about the acceptance of L-tryptophan by SirD. Therefore, L-tryptophan was assayed instead of L-tyrosine. As shown in Figs. 3D and E, a product peak could be clearly detected in the incubation mixture with active SirD, but not in the control assay with heat-inactivated SirD. A relative activity of 8.1 % of that of L-tyrosine was observed for L-tryptophan (Table 1). For structural elucidation, the enzymatic product of SirD with L-tryptophan was isolated and subjected to ^{1}HNMR analysis. It could be shown that the NMR data of the enzymatic product of SirD with L-tryptophan corresponded very well to those of 7-DMATS with L-tryptophan (data not shown) (Kremer *et al.,* 2007). This proved unequivocally that SirD catalysed, besides an O-prenylation of L-tyrosine, also a C-prenylation of L-tryptophan.

Biochemical properties and kinetic parameters of SirD

Indole prenyltransferases from fungi were independent of the presence of metal ions for their prenyl transfer reactions (Steffan *et al.,* 2009). Similar behaviour was also observed for SirD. Testing of different metal ions showed that they were not essential for the prenyltransferase activity of SirD. Even in the presence of 5 mM of the chelating agent EDTA, a relative activity of 87.8 % was observed in comparison to the incubation without additives. A relative activity of 136.6 % of that of the incubation mixture without additives was detected in the presence of 5 mM Ca^{2+}.

The SirD reaction apparently followed Michaelis-Menten kinetics. The K_M values were determined from Lineweaver-Burk plots as 0.13 mM for L-tyrosine and 0.17 mM for DMAPP, respectively. The maximum reaction velocity was 0.58 µmol $min^{-1}.mg^{-1}$, corresponding to a turnover number of 1.0 s^{-1}. The K_M value for L-tryptophan was determined as 0.23 mM, slightly higher than that of L-tyrosine. The maximum reaction velocity for L-tryptophan was determined as 0.035 µmol $s^{-1}.mg^{-1}$, corresponding to a turnover number of

0.06 s^{-1}, being only 6 % of that of L-tyrosine. This proved that L-tyrosine rather than L-tryptophan is the physiological substrate of SirD.

DISCUSSION

In this study we cloned and overexpressed *sirD* from the biosynthetic gene cluster of sirodesmin PL in a heterologous host and proved its function by biochemical characterisation. SirD was unequivocally shown to catalyse the prenylation of L-tyrosine and represents therefore very likely the first pathway-specific enzyme in the biosynthesis of sirodesmin PL (Fig. 1). The enzymatic product of SirD, *i.e.* 4-O-dimethylallyl-L-tyrosine, would then undergo condensation with L-serine catalysed by the non-ribosomal peptide synthetase SirP (Fig. 1). However, it could not be excluded that L-tyrosine would be firstly transferred to SirP and the enzyme-bound form of L-tyrosine is then prenylated. It is also possible that the enzyme-bound linear dipeptide L-Tyr-L-Ser is the true substrate of SirD. Cyclo-L-Tyr-L-Ser was not accepted by SirD as an aromatic substrate, which is in contrast to the results obtained by feeding experiment with ^{14}C-labelled cyclo-L-Tyr-L-Ser in *Phoma lingam* (Ferezou *et al.,* 1980). In that experiment, cyclo-L-Tyr-L-Ser was well incorporated into sirodesmin PL molecule. The reason for these confusing results is unknown. Additional prenyltransferases in the fungus, which accept cyclo-L-Tyr-L-Ser as prenylation substrate, would give an explanation for this phenomenon.

The enzymatic product of L-tyrosine was unequivocally identified as 4-O-dimethylallyl-L-tyrosine by ^{1}HNMR analysis. This demonstrated that SirD forms an O-C bond between a tyrosine and the prenyl donor DMAPP, which differed clearly from those of known indole prenyltransferases from *Aspergillus* and *Neosartorya*. These enzymes catalyse the C-C or N-C bond formation between DMAPP and indole moieties of diverse substances (Ding *et al.,* 2008; Grundmann *et al.,* 2008; Grundmann & Li 2005; Kremer *et al.,* 2007;

Unsöld & Li 2005; Unsöld & Li 2006; Yin *et al.*, 2007). Interestingly, SirD shares a sequence similarity of 34 % with 7-DMATS from *A. fumigatus* (Kremer *et al.*, 2007). This is somehow higher than those of 7-DMATS with all of the known indole prenyltransferases (Steffan *et al.*, 2009). 7-DMATS accepted L-tryptophan, but not L-tyrosine as prenylation substrate (Kremer *et al.*, 2007). In contrast, SirD prenylated both L-tyrosine and L-tryptophan. The enzymatic product of SirD with L-tryptophan was proven to be 7-DMAT, identical to the enzymatic product of 7-DMATS. This could explain the higher sequence similarity of the two proteins. This demonstrated also the higher substrate flexibility of SirD in comparison to 7-DMATS. Comparison of the structure of SirD with those of indole prenyltransferases, especially with that of 7-DMATS, would help to understand this substrate flexibility. Unfortunately, only the structure of the 4-dimethylallyltryptophan synthase FgaPT2 was recently resolved (Metzger *et al.*, 2009). Therefore, it is at the moment not possible to get more details about the substrate binding site of SirD. It could be expected that the reaction mechanism of the SirD reaction is similar to those of other prenyltransferases, *i.e.* formation of a dimethylallyl cation, which attacks an electron-rich carbon or a hetero atom (Haagen *et al.*, 2007; Kuzuyama *et al.*, 2005; Metzger *et al.*, 2009). Like known indole prenyltransferases (Steffan *et al.*, 2009), SirD contains no (N/D)DXXD motif in its sequence and the reaction catalysed by SirD is independent of the presence of metal ions. Therefore, it is likely that metal ions such as Mg^{2+} are not involved in the dimethylallyl cation formation, as in the cases of membrane-bound prenyltransferases (Ohara *et al.*, 2009). In contrast, it could be expected that basic amino acids such as lysine and arginine are involved in the binding of pyrophosphate moiety of DMAPP and responsible for the formation of the cation, as demonstrated for indole prenyltransferases (Metzger *et al.*, 2009; Stec *et al.*, 2008). Corresponding residues, *e.g.* K209, K284, R126 and R288, were indeed found in the sequence of SirD.

Taken the product with L-tryptophan in consideration, SirD catalysed both O-C and C-C bond formation, which was also observed for other prenyltransferases. For example, some

prenyltransferases from gram-positive bacteria *Streptomyces, e.g.* NphB and Fnq26a, were reported to catalyse both C- and O-prenylation of various phenolic substances (Haagen *et al.,* 2007; Kumano *et al.,* 2008). Similar phenomenon was also observed for indole prenyltransferases. FtmPT1 from *A. fumigatus* catalysed the C-prenylation of cyclic dipeptides at position 2 and N-prenylation of tryptophan at position 1 of the indole moieties (Li 2009a). CdpNPT and AnaPT catalysed also both C- and N-prenylation (Yin *et al.,* 2009). This capability of prenyltransferases is of special importance to be used as tool for production of prenylated derivatives (Li 2009a; Macone *et al.,* 2009).

Considering the sequence similarity and shared biochemical properties of SirD with known indole prenyltransferases from fungi, *e.g.* their independence of the prenyltransferase reaction of metal ions (Li 2009b; Steffan *et al.,* 2009), the tyrosine O-prenyltransferase SirD has likely a common ancestor with indole prenyltransferases. This is more obviously by inspection of the phylogenetic tree of the known indole prenyltransferases and SirD (Fig. 4). Fig. 4 showed clearly that SirD is phylogenetically well integrated within the known prenyltransferases and 7-DMATS has even a more near relationship to SirD as to other indole prenyltransferases. Therefore, SirD and 7-DMATS could represent a subclass of prenyltransferases and SirD as a transient enzyme in the evolution of the prenyltransferases.

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Table 1 Relative activity of the prenyltransferase activity of SirD towards different aromatic substances.

Substrate	Relative activity (%)
L-tyrosine	100.0
D-tyrosine	12.3
L-3,4-dihydroxyphenylalanine	16.5
L-tryptophan	8.1
L-phenylalanine	< 0.3
4-hydroxybenzoic acid	< 0.3
para-coumaric acid	< 0.3
cyclo-L-Tyr-L-Ser	< 0.3
cyclo-L-Tyr-L-Pro	< 0.3
cyclo-L-Tyr-L-Trp	< 0.3
cyclo-L-Trp-Gly	< 0.3

The assays (100 µl) contained 2 mM of aromatic substrates, 1 mM of DMAPP, 5 mM of $CaCl_2$ and 48 nM of purified SirD and were incubated at 37 °C for 30 min. A conversion rate of 22.3 % was observed for L-tyrosine and defined as 100 % of relative activity. The data are mean values of two independent assays.

Legends to figures

Figure 1: Proposed biosynthetic pathways of phomamide. A) based on the results from feeding experiments (Ferezou *et al.*, 1980) and B) based on the results of this study.

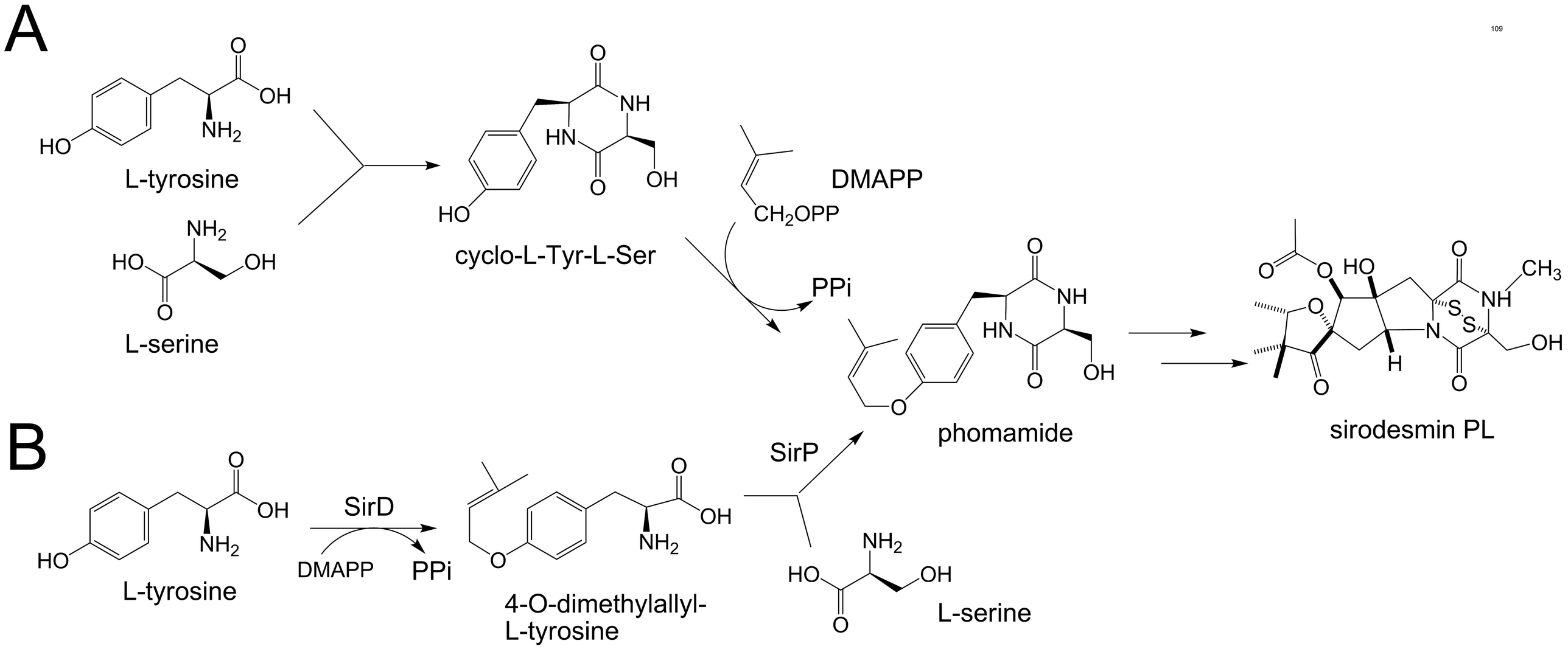
Figure 2: Analysis of the overproduced and purified His_6-SirD. The protein were separated on a 12 % SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue G-250. Lane 1: Molecular mass standard; 2: Total protein before induction; 3: Total protein after induction; 4: Purified His_6-SirD.

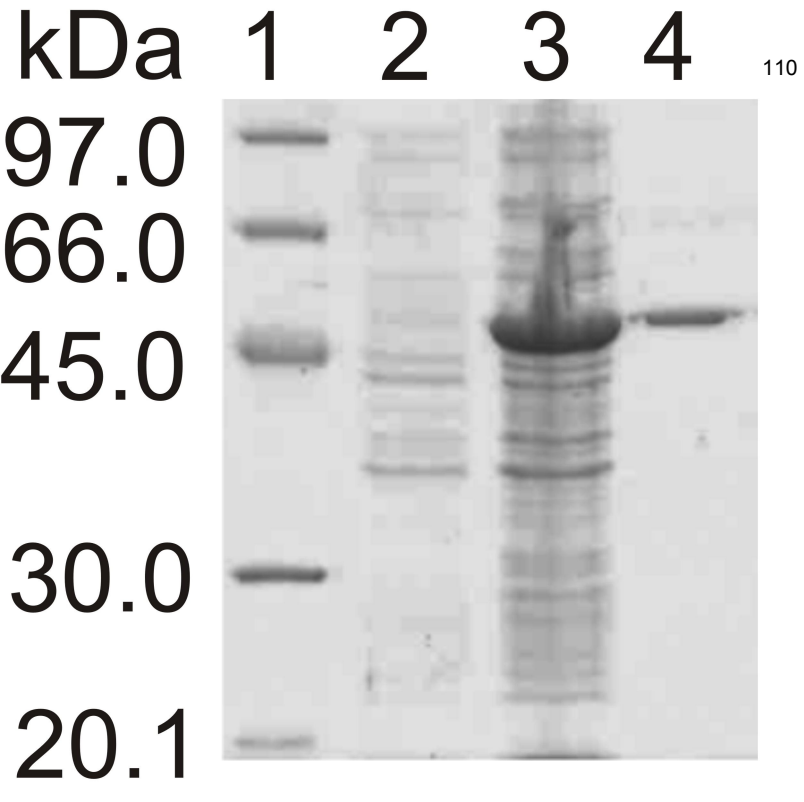
Figure 3: HPLC chromatograms of enzyme assays with His_6-SirD. Incubation of L-tyrosine and DMAPP with active His_6-SirD (A); Incubation of L-tyrosine and DMAPP with heat-inactivated His_6-SirD by boiling of the enzyme solution for 20 min (B); Incubation of cyclo-L-Tyr-L-Ser and DMAPP with active His_6-SirD (C); Incubation of L-tryptophan and DMAPP with active His_6-SirD (D); Incubation of L-tryptophan and DMAPP with heat-inactivated His_6-SirD (E). The incubation mixtures (100 µl) contained 2 mM of aromatic substrates, 1 mM of DMAPP, 5 mM of $CaCl_2$ and 48 nM of purified SirD and were incubated at 37 °C for 60 min. The substances were detected with a Photo Diode Array detector and illustrated at 277 nm.

Figure 4: Phylogenetic relationship of known prenyltransferases from fungi.

ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) was used for alignments of amino acid sequences, average distance BLOSUM62 was selected for tree calculation. FgaPT2 (Acession no. AAX08549), FgaPT1 (XP_756136), FtmPT1 (AAX56314), FtmPT2

(EU622826), 7-DMATS (ABS89001) and CdpNPT (ABR14712) are from *Aspergillus fumigatus*, AnaPT (EAW16181) from *Neosartorya fischeri*, SirD (AAS92554) from *Leptosphaeria maculans*, TdiB (ABU51603) from *A. nidulans,* DMATS-Cs (AAZ29613.1) from IasaF13 and DMATS-Cp (Q6X2E0) from *Claviceps purpurea* and MaPT (EU4200091) from *Malbranchea aurantiaca*


A
L-tyrosine
L-serine
cyclo-L-Tyr-L-Ser
DMAPP
PPi
phomamide
sirodesmin PL
B
L-tyrosine
SirD
DMAPP
PPi
4-O-dimethylallyl-
L-tyrosine
SirP
L-serine


kDa
1
2
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110
97.0
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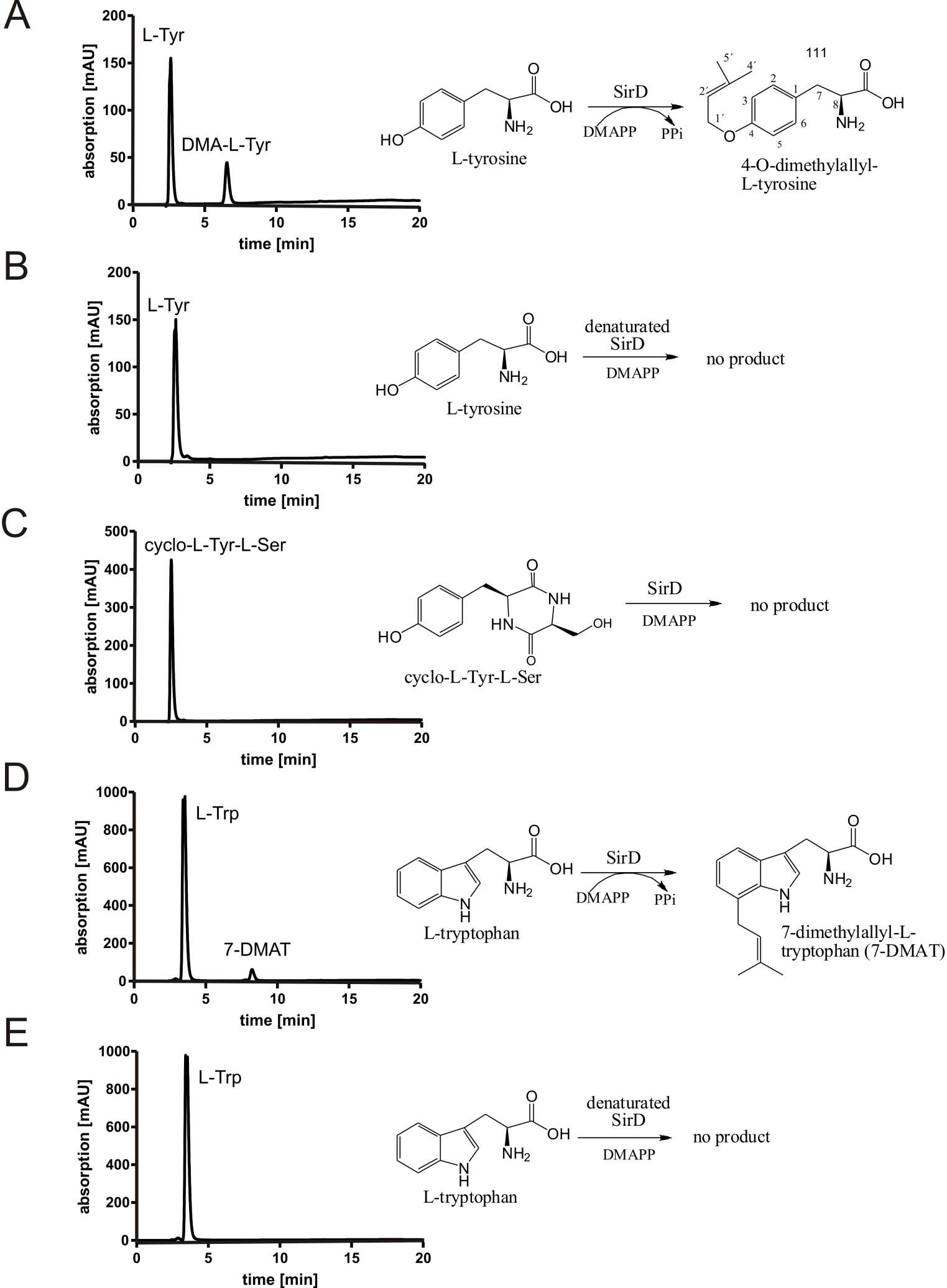
A
L-Tyr
DMA-L-Tyr
absorption [mAU]
time [min]
L-tyrosine
SirD
DMAPP
PPi
4-O-dimethylallyl-
L-tyrosine
B
L-Tyr
absorption [mAU]
time [min]
L-tyrosine
denaturated
SirD
DMAPP
no product
C
cyclo-L-Tyr-L-Ser
absorption [mAU]
time [min]
cyclo-L-Tyr-L-Ser
SirD
DMAPP
no product
D
L-Trp
7-DMAT
absorption [mAU]
time [min]
L-tryptophan
SirD
DMAPP
PPi
7-dimethylallyl-L-
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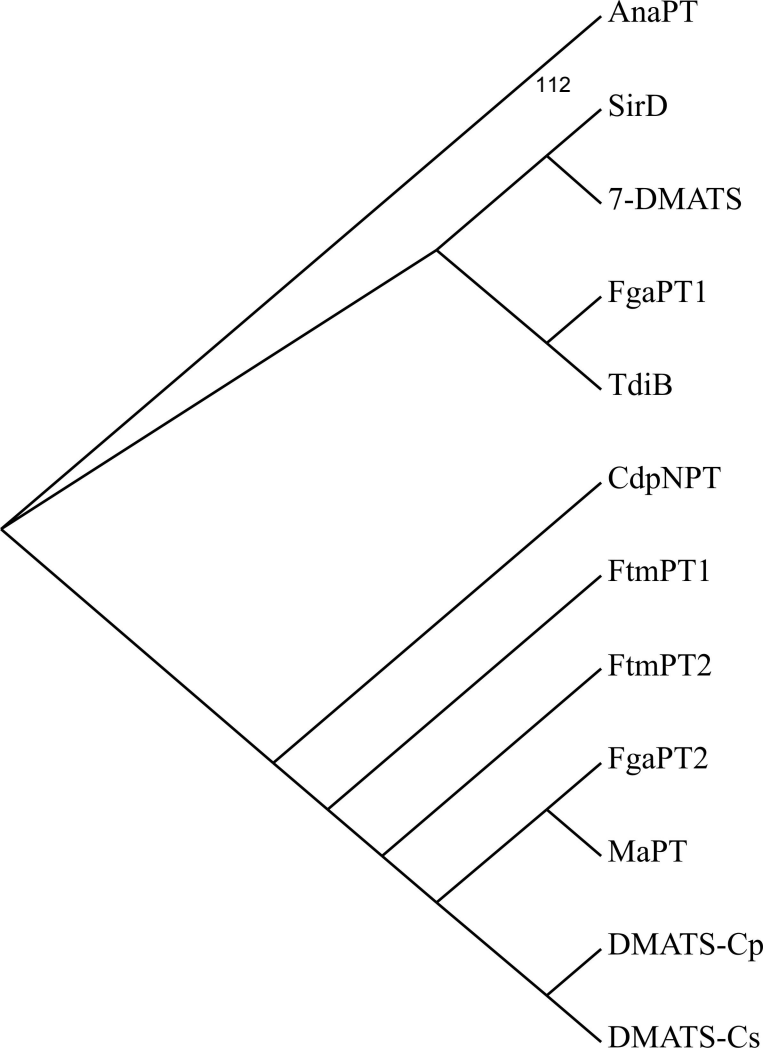
denaturated
SirD
DMAPP
no product


AnaPT
112
SirD
7-DMATS
FgaPT1
TdiB
CdpNPT
FtmPT1
FtmPT2
FgaPT2
MaPT
DMATS-Cp
DMATS-Cs

New insights into the catalytic promiscuity of fungal indole prenyltransferases: metal ions, chelating and reducing agents impact their aminopeptidase activity

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Running title: catalytic promiscuity of fungal indole prenyltransferases

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Abstract

Indole prenyltransferases play a central role in the biosynthesis of prenylated natural products from fungi, among those numerous mycotoxins. In this study, four members of this class of enzymes, FgaPT2 and FtmPT2 from *Aspergillus fumigatus*, TdiB from *Aspergillus nidulans* and AnaPT from *Neosartorya fischeri,* were investigated for aminopeptidase activity, which was previously found for other prenyltransferases (FtmPT1, FgaPT1, CdpNPT, 7-DMATS) from *Aspergillus fumigatus*. Aminopeptidase activity was found for all enzymes tested. The presence of dithiothreitol inhibited aminopeptidase activity of most, but not of all prenyltransferases, while none of the above enzymes was affected by standard peptidase inhibitors. The aminopeptidase activity also differed from the prenyltransferase activity by their response towards chelating agents (EDTA, imidazole, 1,10-phenantroline) as well as metal cations. This study suggests that fungal indole prenyltransferases fall into at least three categories with regard to their aminopeptidase activity i) those inhibited by chelators, but stimulated by Mn^{2+}, ii) those inhibited by both DTT and Mn^{2+}, and iii) a third heterogenous group.

Introduction

Fungal indole prenyltransferases are involved in the biosynthesis of numerous bioactive indole alkaloids (Steffan *et al.,* 2009). These enzymes differ from each other not only by the respective natural substrate and prenylation position, but also by prenylation pattern, i.e. regular or reverse, as well as transfer to carbon- or nitrogen atoms (Li, 2009). However, they share several biochemical properties, e.g. the lacking requirement for metal ions, their strict specificity towards dimethylallyl diphosphate as prenyl donors and their flexibility towards aromatic substrates (Steffan *et al.,* 2009). Recently, an unexpected second catalytic activity was identified for four prenyltransferases (7-DMATS, CdpNPT, FgaPT1 and FtmPT1) from *Aspergillus fumigatus* (Kremer and Li, 2008): these prenyltransferases showed an intrinsic tryptophan aminopeptidase activity with preference towards tryptophan-containing linear dipeptides (Kremer and Li, 2008). To further explore this intriguing phenomenon, we investigated biochemically the aminopeptidase activity of other three indole prenyltransferases, FtmPT2, TdiB and AnaPT, from different Aspergilli implicated in the biosynthesis of diverse mycotoxins, such as fumitremorgin B (Grundmann *et al.,* 2008), terrequinone A (Balibar et al., 2007;Schneider et al., 2008), and acetylaszonalenin (Yin *et al.,* 2009). We also reinvestigated FgaPT2, whose aminopeptidase activity was not observed previously (Kremer and Li, 2008). For all eight enzymes mentioned above, this study establishes differences in the requirements towards reducing agents, chelating agents and metal ions, to exert aminopeptidase activity, and proposes subgroups for fungal indole prenyltransferases.

Results

Peptidase activity of prenyltransferases

To determine their aminopeptidase activity, recombinant and purified TdiB from *A. nidulans,* AnaPT from *Neosartorya fischeri* and FtmPT2 from *A. fumigatus* were incubated with the L-tryptophan-glycine dipeptide (H-L-Trp-Gly-OH) for 16 hr and the incubation mixtures were subsequently analysed by HPLC using a RP-18 column. HPLC analysis showed clearly the formation of tryptophan in all incubation mixtures with active, but not in those with heat-inactivated recombinant proteins (Fig. 1). The identity of the product as tryptophan was confirmed by comparison of the retention time with that of an authentic reference (data not shown). The conversion rates found for FtmPT2, TdiB and AnaPT were 31.0, 22.2, and 44.7 % respectively. This proved unequivocally the aminopeptidase activity of these enzymes. Combined with previous results (Kremer and Li, 2008), seven of the eight tested prenyltransferases (FgaPT1, FtmPT1, CdpNPT, 7-DMATS, FtmPT2, TdiB and AnaPT) showed aminopeptidase activity. The only exception was FgaPT2, whose aminopeptidase activity could not be detected in the previous study (Kremer and Li, 2008).

Inhibition of the aminopeptidase activity by reducing agents

As dithiothreitol (DTT) may inhibit peptidases (Karadzic et al., 2002;Magboul and McSweeney, 1999;Cahan et al., 2007), we reinvestigated the aminopeptidase activity of FgaPT2 and incubated it again with H-L-Trp-Gly-OH, unlike previously, in a DTT-free reaction buffer. As shown in Figs. 1G and 1H, tryptophan formation was clearly detected in the reaction mixture with active FgaPT2, but not in that with heat-inactivated protein. With 3 µg of FgaPT2, 88 % substrate conversion was observed after incubation for 16 hr. This proved clearly that FgaPT2 has aminopeptidase

activity , which was obscured previously (Kremer and Li, 2008) due to the presence of DTT.

This raises the question whether the DTT-mediated inhibition of the aminopeptidase activity is specific for FgaPT2 or a general feature of other fungal prenyltransferases. All eight prenyltransferases mentioned above were therefore incubated with H-L-Trp-Gly-OH in the presence of 10 mM DTT for 1 hr. Incubations without DTT served as controls. Complete inhibition was observed in the incubation mixtures of FgaPT1, FgaPT2, FtmPT2, 7-DMATS, AnaPT and TdiB. Surprisingly, DTT had only a minor effect on CdpNPT and FtmPT1, with relative aminopeptidase activities of 78.9 and 65.7 %, in comparison to those without DTT (Table 1).

We recorded aminopeptidase inhibition as a function of DTT concentration for 7-DMATS as a prototypical fungal prenyltransferase (Fig. 2). More than 50 % inhibition was observed at 0.2 mM DTT. Only 1.5 % residual activity was found at 5 mM. In contrast, the prenyltransferase activity of 7-DMATS was not significantly inhibited, even at DTT concentrations of 20 mM (data not shown).

Effects of other reducing agents on aminopeptidase activity were also investigated. Glutathione (GSH) and its oxidized form glutathione disulfide (GSSG) represent the most abundant redox system in eukaryotic cells and play an important role in many physiological processes (Meister and Anderson, 1983). Incubation of H-L-Trp-Gly-OH in the presence of 5 mM GSH completely inhibited peptidase activity of all eight tested prenyltransferases (Table 1).

Effect of various inhibitors on the aminopeptidase activity

Indole prenyltransferases show no sequence similarity to known peptidases. To compare their biochemical properties, all eight indole prenyltransferases were

incubated in the presence of different aminopeptidase inhibitors (Karadzic et al., 2002;Cahan et al., 2007;Ito et al., 2003;Bolumar et al., 2008) (Table 1). Only insignificant changes of the peptidase activity were observed in the presence of the cysteine protease inhibitors leupeptin, E-64 and EST (Barrett *et al.,* 1982). Similar results were obtained with the chymotrypsin protease inhibitor TPCK (Stoppler *et al.,* 1996) (Table 1).

Influence of metal ions and chelating agents on the peptidase activity of prenyltransferases

Peptidase activity of the prenyltransferases is partially or completely inhibited by addition of the chelator EDTA to the reaction mixtures. Metal ions such as Mn^{2+} have heterogenous effects on the peptidase activity (Kremer and Li, 2008). In this study, we tested the effect of FtmPT2, FgaPT2, AnaPT and TdiB on the presence of different ions and EDTA. Incubations without additives were used as controls. Addition of EDTA to a final concentration of 5 mM to the reaction mixtures of FgaPT2, FtmPT2 and TdiB resulted in complete inhibition of the peptidase activity, while the peptidase activity of AnaPT was still detectable and decreased to 17 % compared to the control without additives. Together with a previous study (Kremer and Li, 2008), the results show that the aminopeptidase activity of the indole prenyltransferases could be completely (CdpNPT, FtmPT1, FgaPT2, FtmPT2 and TdiB), or strongly (7-DMATS and AnaPT) inhibited by 5 mM EDTA (Table 2). In contrast, the aminopeptidase activity of FgaPT1 was less inhibited by EDTA, and 43 % of the activity remained in the presence of 5 mM EDTA. Another metal chelator, 1,10-phenantroline (Bolumar *et al.,* 2008) showed overall similar, yet somewhat stronger effects than EDTA (Table 2). In the presence of 5 mM of 1,10-phenantroline,

the aminopeptidase activity of FgaPT1 was reduced to 16.7 % of that of the control assay (Table 2). This demonstrated the absolute requirement of metal ions for the peptidase activity of the indole prenyltransferases.

Pertaining to their peptidase activity, these four enzymes responded heterogeneously to the presence of 5 mM mono- and bivalent metal cations: more than 140 % activity, compared to controls without additive, was detected for CdpNPT with Mn^{2+}, for FtmPT1 and FgaPT2 with Mn^{2+}, Mg^{2+} and Ca^{2+}, and for FgaPT1 with Ca^{2+}. In the presence of 5 mM of Mn^{2+}, 207, 422 and 591 % of the activities of the control assays were observed for FgaPT2, FtmPT1 and CdpNPT, respectively (Table 2). In contrast, almost no increase of the aminopeptidase activity of FtmPT2, AnaPT, TdiB and 7-DMATS was detected after addition of different metal ions (Table 2). This indicates that metal ions are bound in some cases more tightly in the active centres or even different metal ions are utilized for catalysis. The latter hypothesis is more plausible if the results of the aminopeptidase activity in the presence of imidazole are taken into consideration. Incubation of prenyltransferases with H-L-Trp-Gly-OH in the presence of 100 mM imidazole resulted in complete inhibition of FgaPT1, FtmPT2, AnaPT, TdiB and 7-DMATS (Table 2), while the aminopeptidase activity of CdpNPT, FtmPT1 and FgaPT2 was only reduced to 35, 65 and 83 % compared with control reactions without additives.

Influence of the peptidase activity by site-directed mutagenesis

The observed differences of the indole prenyltransferases mentioned above were supported by results obtained with mutated prenyltransferases. In course of the search for binding sites of dimethylallyl diphosphate involved in the prenyltransferase activities, several conserved amino acids were mutated (Stec *et al.,* 2008). One of those was a conserved arginine residue (Arg292 in FtmPT1, Arg257 in FgaPT2 and

Arg311 in 7-DMATS). These residues were mutated to glycine by site-directed mutagenesis, respectively, resulting in the mutated proteins FtmPT1_R292G, FgaPT2_R257G and 7-DMATS_R311G. The mutated proteins were incubated with H-L-Trp-Gly-OH by using the respective nonmutated proteins as control. The aminopeptidase activities of FtmPT1_R292G and FgaPT2_R257G were increased to 186 and 281 % compared to controls, respectively, while the activity of 7-DMATS_R311G was reduced to 24 % of that of 7-DMATS.

Discussion

This study reported aminopeptidase activity of four additional indole prenyltransferases. As all of the tested indole prenyltransferases showed peptidase activity towards the linear dipeptide H-L-Trp-Gly-OH, this second catalytic activity might represent a general feature, whose physiological reason, however, remains elusive. In previous and present studies, we have demonstrated that the aminopeptidase activity of all of the tested enzymes was inhibited by the chelating agents EDTA and 1,10-phenantroline, although with somewhat different intensities (Table 2). This finding contrasts the results obtained for the prenyltransferase activities, which were not inhibited by EDTA (Steffan et al., 2009;Li, 2009), possibly indicating that the prenyltransferase and aminopeptidase activities have different active centres. Alternatively, the same active centre might be involved in both activities, yet adapted to aminopeptidase activity by binding appropriate metal cations.

Except CdpNPT and FtmPT1, the aminopeptidase activity of indole prenyltransferases was completely inhibited by the reducing agent DTT (Table 1). This finding is in accordance with results observed for leucine aminopeptidases from *Streptomyces hygroscopicus* (Karadzic *et al.,* 2002) and *Bacillus thuringiensis*

israelensis (Cahan *et al.,* 2007) as well as for a dipeptidase from *Lactobacillus curvatus* (Magboul and McSweeney, 1999). In the presence of 5 mM glutathione, the aminopeptidase activity of all eight indole prenyltransferases was completely inhibited, while prenyltransferase activity was not affected by one of the reducing agents. The reason for the modulation of aminopeptidase activity by the presence and absence of reducing agents is unknown. The reducing environment in the cell or in cell compartments maintains prenyltransferase activity in the context of mycotoxin biosynthesis, whereas the reducing enviroment suppresses aminopeptidase activity so that uncontrolled hydrolysis of the endogenic peptides by these enzymes might be avoid. However, if aminopeptidase activity is inhibited under certain conditions, for hydrolysis of endogenic or exogenic peptides an oxidative environment outside the cell or in a particular compartment will form a functional aminopeptidase.

Based on our data, fungal indole prenyltransferases may be categorised in different subgroups (Table 3). The first group includes CdpNPT and FtmPT1. The aminopeptidase activity within this group is completely inhibited by EDTA and 1,10-phenantroline, but only slightly by imidazole and by DTT at concentrations used in this study. Addition of metal ions, especially Mn^{2+}, strongly enhanced the enzymatic activity. In contrast, the aminopeptidase activity of the second group consisting of FtmPT2, AnaPT, TdiB and 7-DMATS was completely or partially (more than 80 %) inhibited by all four agents. Addition of metal ions did not result in increasing of enzymatic activity, and Mn^{2+} decreased aminopeptidase activity. FgaPT2 and FgaPT1 could not be classified into these two groups and fall in an intermediate third group that combines features from the former two. In the case of FgaPT2, the aminopeptidase activity was strongly inhibited by EDTA, 1,10-phenantroline and DTT, but less by imidazole. Addition of metal ions such as Mn^{2+}, Mg^{2+} and Ca^{2+} resulted in a comparable and moderate increase of enzyme activity. In the case of

FgaPT1, the aminopeptidase was strongly inhibited by 1,10-phenantroline, DTT and imidazole, but only weakly by EDTA. Mg^{2+} and Ca^{2+} slightly enhanced the enzyme activity. This initial categorization warrants further studies, also in front of an evolutionary background, to further refine subgroups within fungal indole prenyltransferases.

Materials and Methods

Chemicals

H-L-Trp-Gly-OH was purchased from Bachem (Bubendorf, Switzerland). Inhibitors were purchased from Merck (Darmstadt, Germany).

Bacterial strains, plasmids and culture conditions

Escherichia coli XL1 Blue MRF´ and BL21(DE3)pLysS were used for overexpression experiments (Sambrook and Russell, 2001). Carbenicillin or kanamycin (50 µg/mL) was used for selection of recombinant *E. coli* strains.

Protein overexpression, purification and analysis

Overproduction of His_8-tagged FgaPT2 was carried out in *E. coli* BL21(DE3)pLysS using the expression plasmid pIU18 with kanamycin as selection marker as described previously (Steffan *et al.,* 2007). The protein was purified on Ni-NTA-agarose and stored in 50 mM Tris-HCl (pH 7.5) containing 15 % (v/v) glycerol (Steffan *et al.,* 2007). Other proteins were overproduced in *E. coli* XL1 Blue MRF´ using carbenicillin as selection marker and purified to near homogeneity as described previously (Unsöld and Li, 2006;Grundmann et al., 2008;Schneider et al., 2008;Kremer et al., 2007;Yin et al., 2007;Grundmann and Li, 2005;Yin et al., 2009).

Proteins were analysed by SDS-PAGE according to the method of Laemmli (1970) and stained with Coomassie Brilliant Blue G-250.

Assays for peptidase activity

For quantitative determination of the aminopeptidase activity, the standard reaction mixtures (100 µl) were done in duplicate and contained 50 mM Tris-HCl (pH 7.5), 2 mM H-L-Trp-Gly-OH, 1.5 % (v/v) glycerol and 3 µg of purified recombinant proteins. In the case of FgaPT2, FtmPT1 and CdpNPT, $MnCl_2$ at a final concentration of 5 mM was added to the incubation mixtures. After incubation for 1 hr at 37° C, the reaction was stopped with 100 µl methanol. The proteins were removed by centrifugation at 13,000 x *g,* for 10 min. The enzymatic products were analysed by HPLC under the conditions described below.

Assays for determination of effects of inhibitors

To determine the effect of different inhibitors, the standard reaction mixtures (100 µl) were supplemented with one of the inhibitors leupeptin, E-64, EST or TPCK, in concentrations between 0.5 and 50 µM according to the manufacturer's instruction. After incubation for 1 hr at 37 °C, the reaction was stopped with 100 µl methanol. To determine the effect of DTT and glutathione on the enzymatic activity, 7-DMATS, FtmPT1, FtmPT2, CdpNPT, FgaPT1, FgaPT2, TdiB and AnaPT were incubated in the presence of 10 mM DTT or 5 mM glutathione for 1 hr at 37 °C, the reaction was stopped with 100 µl methanol. 7-DMATS was also incubated in the presence of 0.1-20 mM of DTT. For determination of the effect of DTT on the prenyltransferase activity 7-DMATS was incubated in the presence of 0.1- 20 mM of DTT and 1 mM L-tryptophan and 1mM DMAPP. To determine the effect of imidazole, the proteins were incubated in the presence of 100 mM imidazole for 1 hr at 37 °C, then the reaction was stopped by adding 100 µl methanol.

Site-directed mutagenesis

Site-directed mutagenesis was carried out by using the QuikChange® II Site-Directed Mutagenesis Kit from Stratagene (Amsterdam, Netherlands) according to the manufacturer´s protocol. PCR amplification was carried out on an iCycler from BioRad (Munich, Germany). The primers used for mutagenesis were synthesized by MWG-Biotech (Ebersberg, Germany). The template plasmids pIU18 containing *fgaPT2* in pHis8 (Jez et al., 2000), pAG12 containing *ftmPT1* in pQE70, and pLW40 containing *7-dmats* in pQE60 were described previously (Grundmann and Li, 2005;Steffan et al., 2007;Kremer et al., 2007) and used as templates for PCR amplification of mutated *fgaPT2*, *ftmPT1* and *7-dmats*, respectively.

HPLC analysis

The enzymatic products were analysed by HPLC on an Agilent HPLC Series 1200 equipped with an Eclipse XDB-C18 column (4.6 x 150 mm, 5 µm, Agilent), at a flow rate of 1 ml min^{-1}. Solvent A was 0.5 % (v/v) trifluoroacetic acid in water, solvent B 0.5 % (v/v) trifluoroacetic acid in methanol. For separation, a linear gradient of 30 - 70 % B within 15 min was applied. The column was then washed with 100 % B and equilibrated with 30 % B, for 5 min. Detection was carried out by a Photo Diode Array detector.

Acknowledgements

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Table 1: Influence of peptidase inhibitors as well as reducing agents on the aminopeptidase activity of fungal prenyltransferases

Additive	Relative activity (%)							
	CdpNPT	FtmPT1	FgaPT2	FgaPT1	FtmPT2	AnaPT	TdiB	7-DMATS
No additive	100 ± 0.3	100 ± 2.3	100 ± 2.0	100 ± 0.5	100 ± 3.1	100 ± 1.5	100 ± 3.7	100 ± 7.0
E-64	100 ± 2.0	95.4 ± 2.9	103.8 ± 3.8	99.0 ± 1.5	107.7 ± 1.5	126.5 ± 3.0	107.4 ± 3.7	91.2 ± 1.7
EST	99.7 ± 0.4	96.0 ± 0.4	96.3 ± 3.8	98.9 ± 1.5	106.2 ± 0.5	114.1 ± 3.5	98.2 ± 5.5	99.1 ± 4.4
Leupeptin	92 ± 1.0	92.2 ±0.3	96.0 ± 6.0	101.6 ± 2.1	103.1 ± 3.1	123.5 ± 5.9	76.9 ± 5.9	95.1 ± 2.6
TPCK	86.7 ± 2.7	94.1 ± 2.3	82.5 ± 0.1	75.9 ± 3.7	110.8 ± 1.6	105.3 ± 6.5	103.7 ± 7.4	134.0 ± 4.5
DTT	78.9 ± 2.8	65.7 ± 1.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2
Glutathione	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2

A conversion rate of 15.1 % for CdpNPT, 5.1 % for FtmPT1, 10.8 % for FgaPT2, 8.5 % for FgaPT1, 5.3 % for FtmPT2, 6.9 % for AnaPT, 4.5 % for TdiB and 11.5 % for 7-DMATS was observed for assays without inhibitors and defined as 100 % of relative activity. The final concentration of the additives was as following: E-64: 5 µM; EST: 50 µM; leupeptin: 50 µM; TPCK: 50 µM; DTT: 10 mM and reduced glutathione: 5 mM

Table 2: Influence of metal ions or chelating agents on the peptidase activity of fungal prenyltransferases

	Relative activity (%)							
Additive	CdpNPT*	FtmPT1*	FgaPT2	FgaPT1*	FtmPT2	AnaPT	TdiB	7-DMATS*
No additive	100 ± 0.8	100 ± 1.6	100 ± 7.6	100 ± 4.1	100 ± 9.8	100 ± 2.7	100 ± 6.9	100 ± 4.1
Ca^{2+}	69.2 ± 6.7	**144.1 ± 2.3**	**180.0 ± 2.8**	**144.2 ± 8.5**	49.7 ± 3.9	73.2 ± 7.7	90 ± 5.1	102.7 ± 2.9
Mg^{2+}	104.0 ± 6.0	**155.2 ± 3.8**	**149.0 ± 1.2**	137.9 ± 1.8	109.8 ± 12.9	110.9 ± 0.6	93.4 ± 5.4	103.5 ± 1.2
Mn^{2+}	**591.2 ± 5.2**	**421.6 ± 1.7**	**207.4 ± 8.7**	69.7 ± 1.1	5.7 ± 0.3	27.1 ± 4.9	51.7 ± 3.3	84.0 ± 2.0
Co^{2+}	46.9 ± 1.5	130.4 ± 2.4	52.1 ± 1.8	32.3 ± 0.3	9.8 ± 1.6	13.2 ± 3.2	≤ 0.2	10.2 ± 1.3
Fe^{2+}	34.2 ± 2.8	42.8 ± 0.5	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2
Na^{+}	94.8 ± 0.8	108.6 ± 2.0	107.1 ± 3.8	108.2 ± 5.1	98.0 ± 1.2	99.2 ± 5.1	90.9 ± 2.2	97.0 ± 3.9
K^{+}	98.7 ± 4.3	86.5 ± 4.3	60.0 ± 8.4	117.9 ± 5.9	27.1 ± 2.0	100.4 ± 0.4	64.2 ± 0.3	103.7 ± 2.7
EDTA	≤ 0.2	≤ 0.2	≤ 0.2	43.1 ± 2.1	≤ 0.2	17.0 ± 1.6	≤ 0.2	11.6 ± 0.2
1,10-Phen-antroline	≤ 0.2	≤ 0.2	≤ 0.2	16.7 ± 3.3	≤ 0.2	8.3 ± 0.2	≤ 0.2	6.1 ± 0.9
Imidazole	35.3 ± 1.5	64.6 ± 1.2	82.7 ± 2.3	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2	≤ 0.2

*Data for metal ions and EDTA were adopted from reference (Kremer and Li, 2008).

The imidazole concentration in the incubation mixtures was 100 mM. The final concentration of other additives was 5 mM. Activities higher than 140 % of the control incubations without additives were highlighted as bold numbers.

Table 3: Overview of the indole prenyltransferases from fungi

Enzyme	**CdpNPT**	**FtmPT1**	**FgaPT2**	**FgaPT1**	**FtmPT2**	**AnaPT**	**TdiB**	**7-DMATS**
Source	*A. fumigatus*	*A. fumigatus*	*A.fumigatus*	*A. fumigatus*	*A.fumigatus*	*N. fischeri*	*A.nidulans*	*A.fumigatus*
Biosynthetic gene cluster	unknown	fumitremorgin	fumigaclavine	fumigaclavine	fumitremorgin	acetylaszonalenin	terrequinone A	unknown
Substrate for prenylation	cyclic dipeptide	cyclic dipeptide	L-tryptophan	fumigaclavine A	12,13-dihydroxyfumitremorgin C	cyclic dipeptide	didemethylasterriquinone D	L-tryptophan
Prenylation mode	reverse N - prenylation	regular C – prenylation	regular C - prenylation	reverse C - prenylation	regular N - prenylation	reverse C - prenylation	reverse C - prenylation	regular C - prenylation
Influence of additives on aminopeptidase activity by using H-L-Trp-Gly-OH as substrate*								
Inhibition by 5 mM DTT (%)	21	34	100	100	100	100	100	100
Inhibition by 100 mM imidazole (%)	65	35	17	100	100	100	100	100
Activity in the presence of 5 mM Mn^{2+} (%)	591	422	207	70	6	27	52	84
Inhibition by 5 mM EDTA (%)	100	100	100	57	100	83	100	88

*The relative activities are obtained by comparison of the incubations containing additives with that of incubations without additives.

Legends to figures

Fig. 1: Hydrolysis of H-L-Trp-Gly-OH by different prenyltransferases. The incubation mixtures contained 2 mM substrate, 3 µg of the purified protein. Mn^{2+} ions of 5 mM were added to the mixtures in the case of FgaPT2. Incubations were carried out at 37 °C for 16 hr. Detection was at 277 nm.

Fig. 2: Dependence of the peptidase activity (■) and the prenyltransferase activity (▲) of 7-DMATS on the DTT concentrations.

Fig. 1

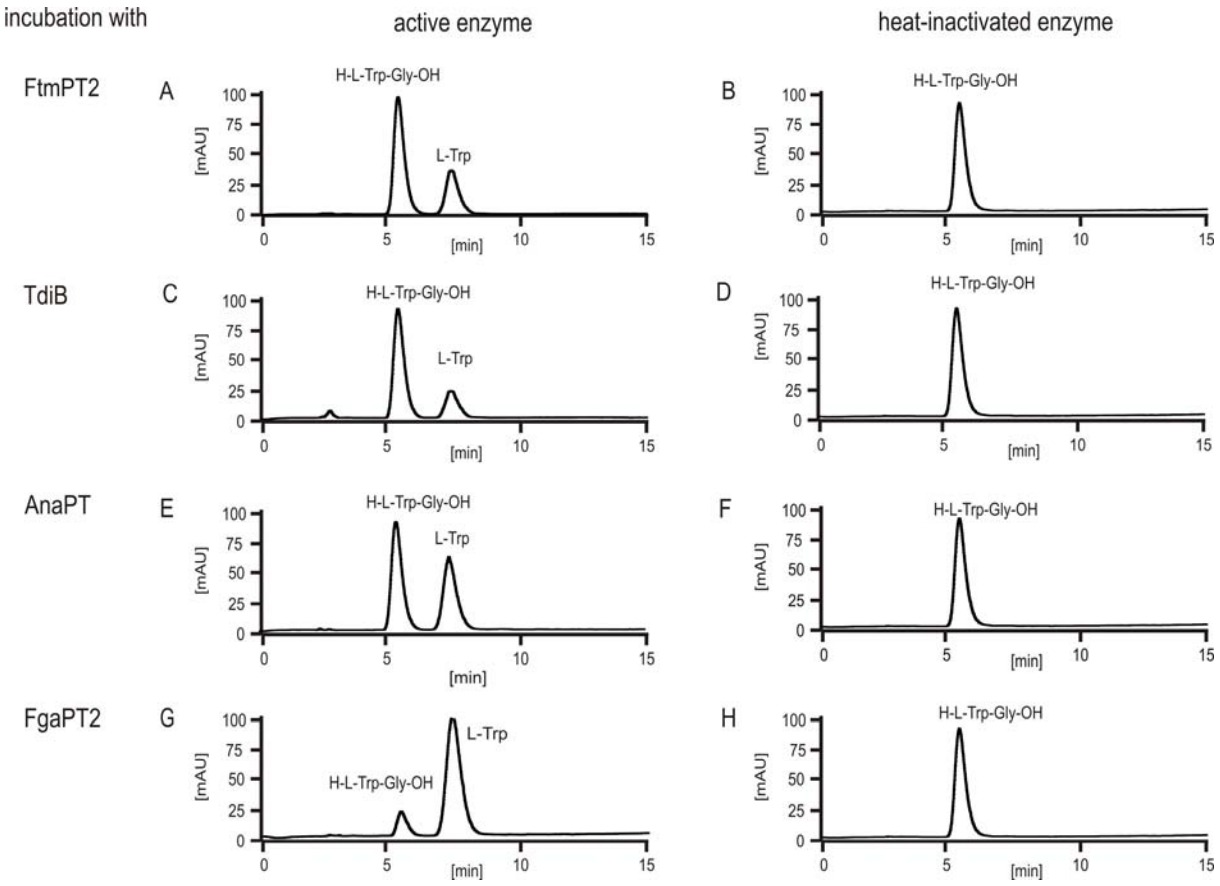
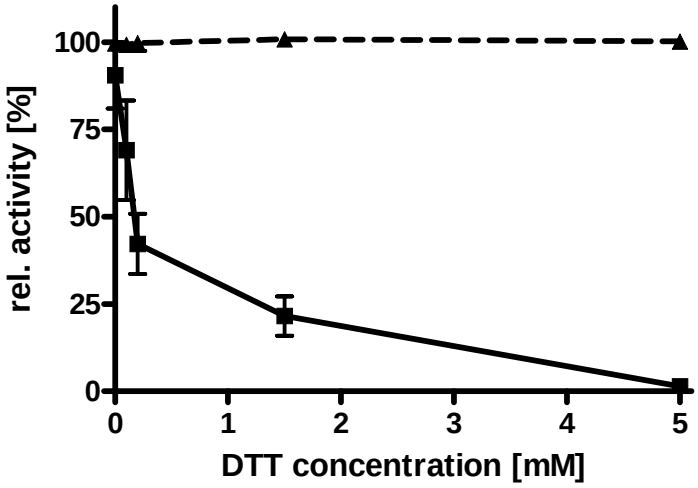

incubation with
active enzyme
heat-inactivated enzyme
FtmPT2
TdiB
AnaPT
FgaPT2
A
B
C
D
E
F
G
H
H-L-Trp-Gly-OH
L-Trp
[mAU]
[min]
100
75
50
25
0
0
5
10
15

Fig. 2



4.2 ^{1}H-NMR -Spektren der isolierten enzymatischen Produkte

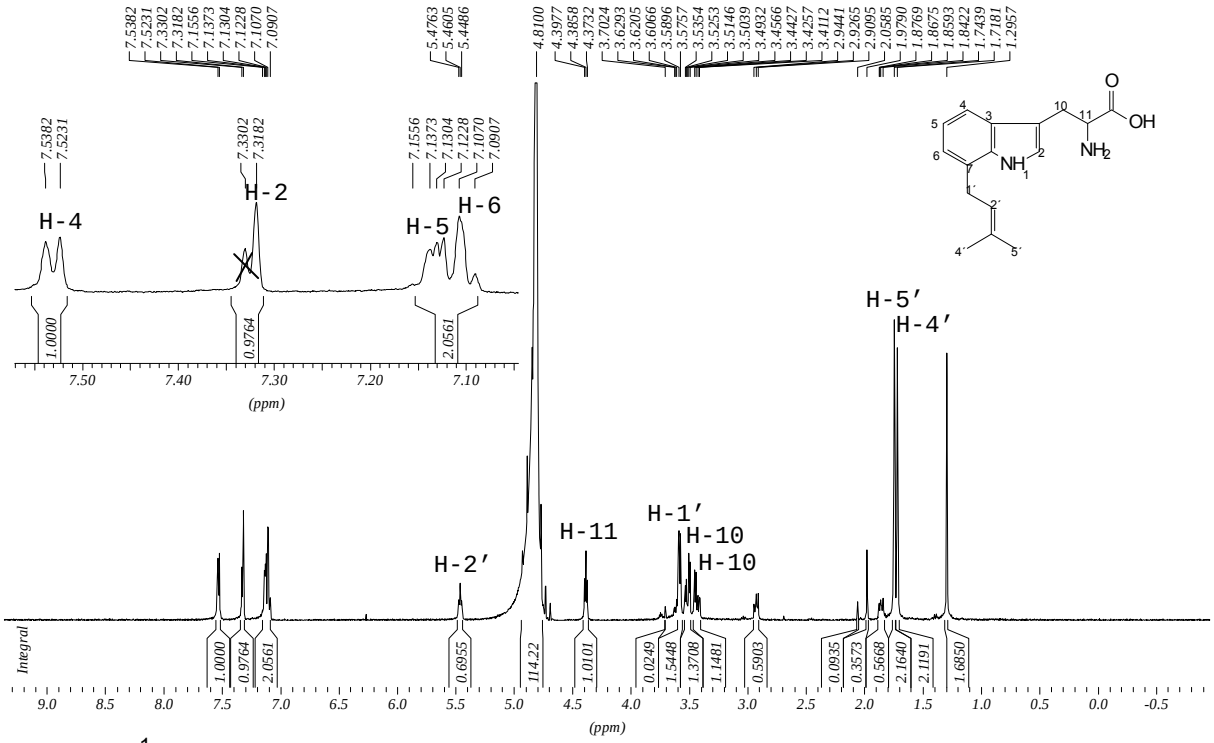


Abb. 4.1: ^{1}H-NMR von 7-Dimethylallyltryptophan, aufgenommen in 0,1 M DCl bei 500 MHz

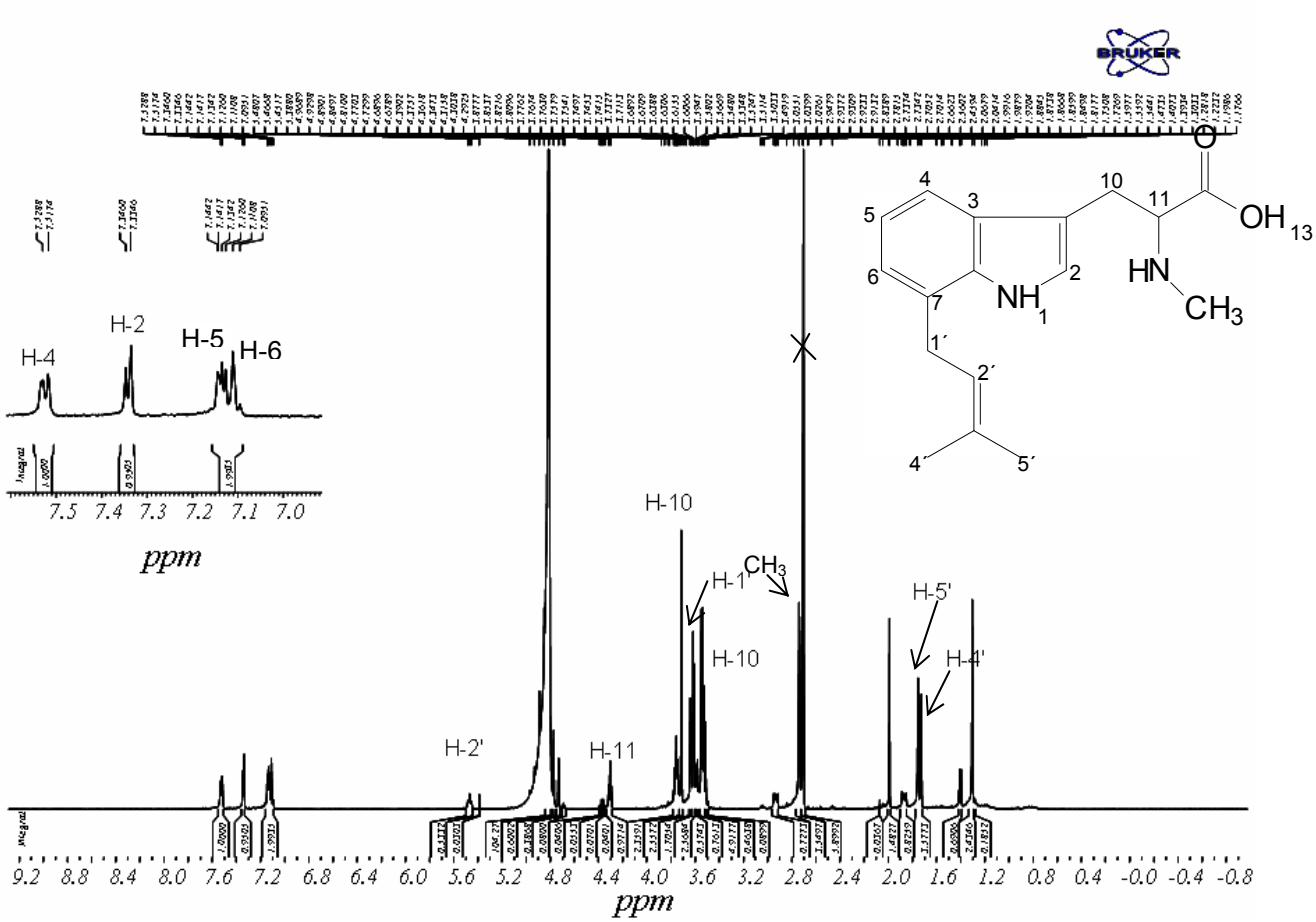


Abb. 4.2: ^{1}H-NMR von 7-DMA-Abrin, aufgenommen in 0,1 M DCl bei 500 MHz

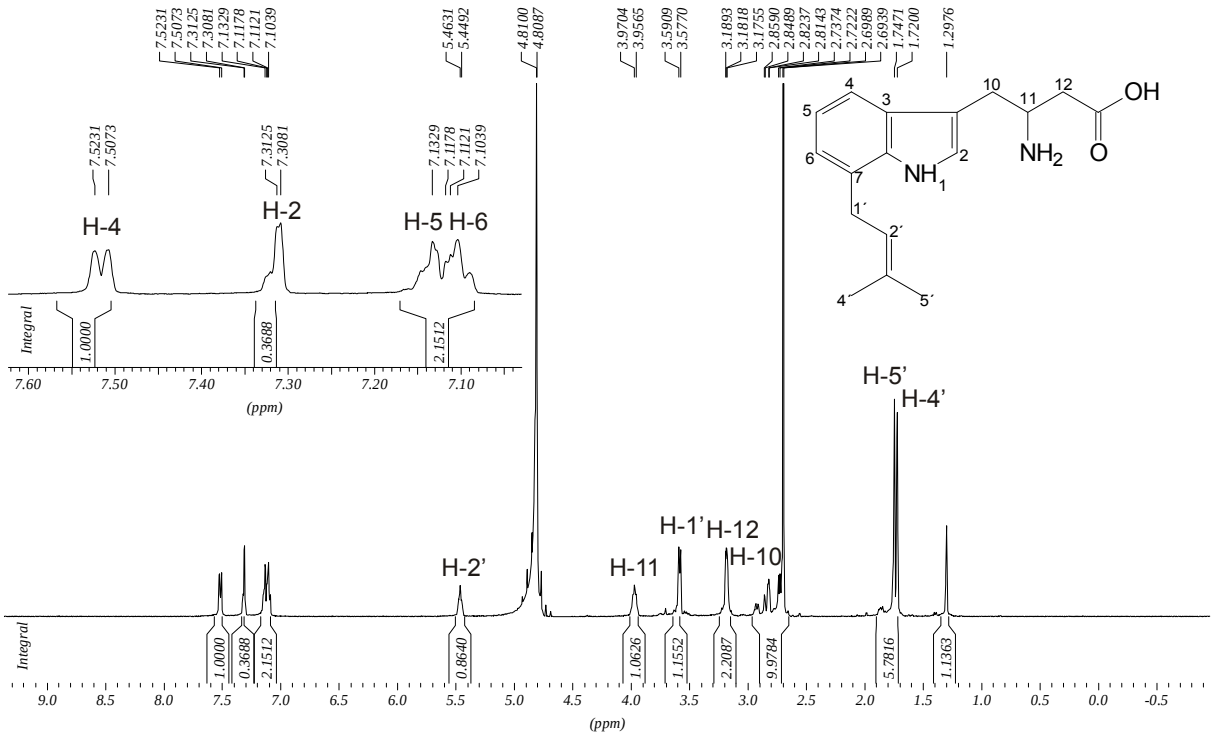


Abb. 4.3: ^{1}H-NMR von 7-DMA-β-Homotryptophan, aufgenommen in 0,1 M DCl bei 500 MHz

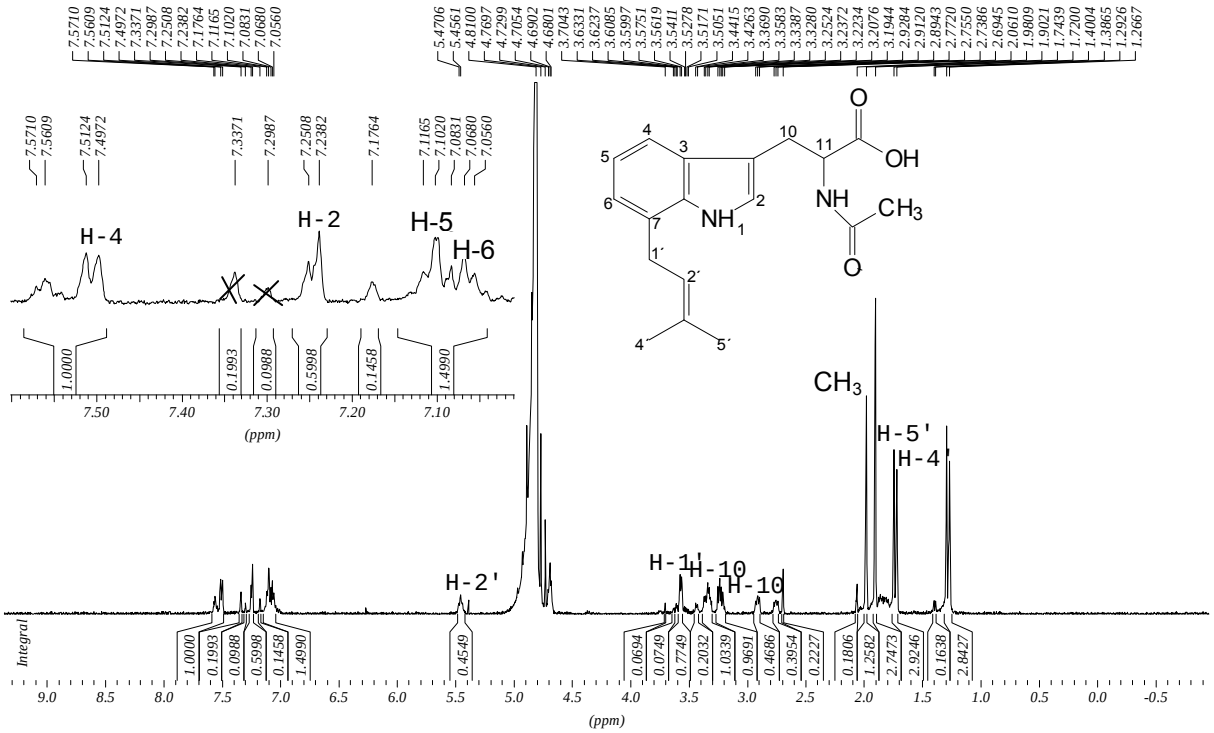


Abb. 4.4: ^{1}H-NMR von *N*-Acetyl-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

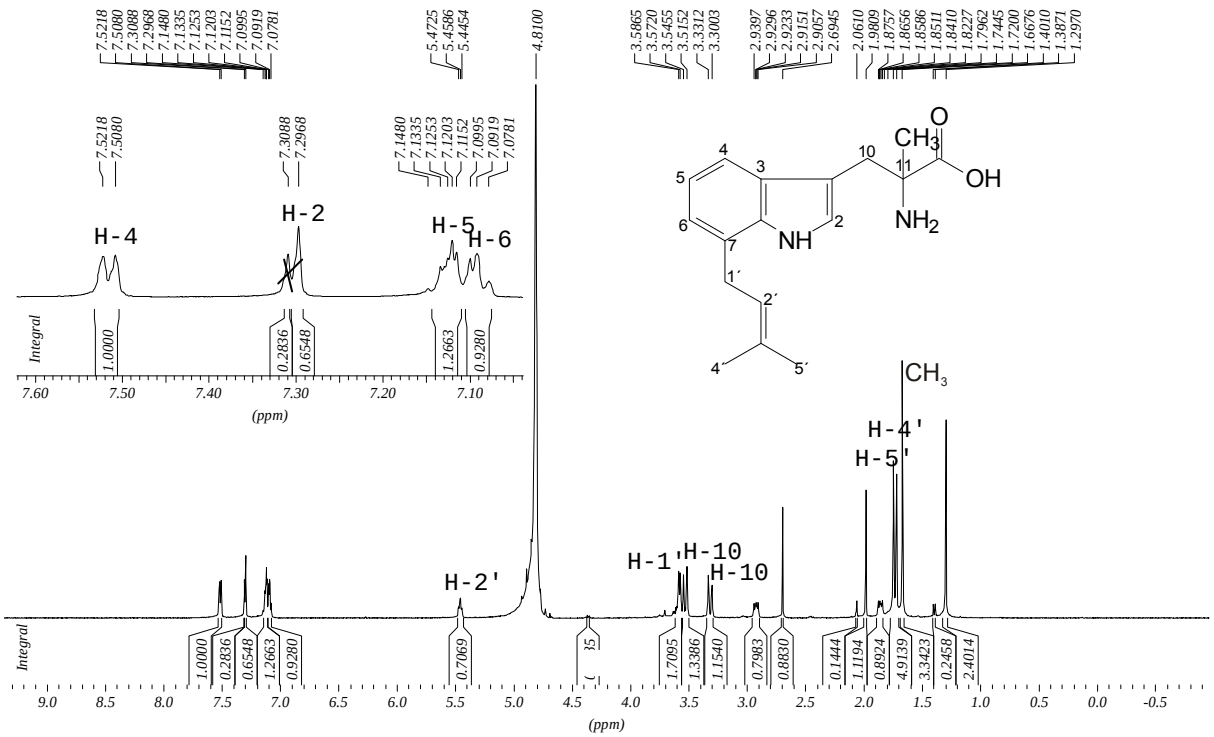


Abb. 4.5: ^{1}H-NMR von 11-Methyl-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

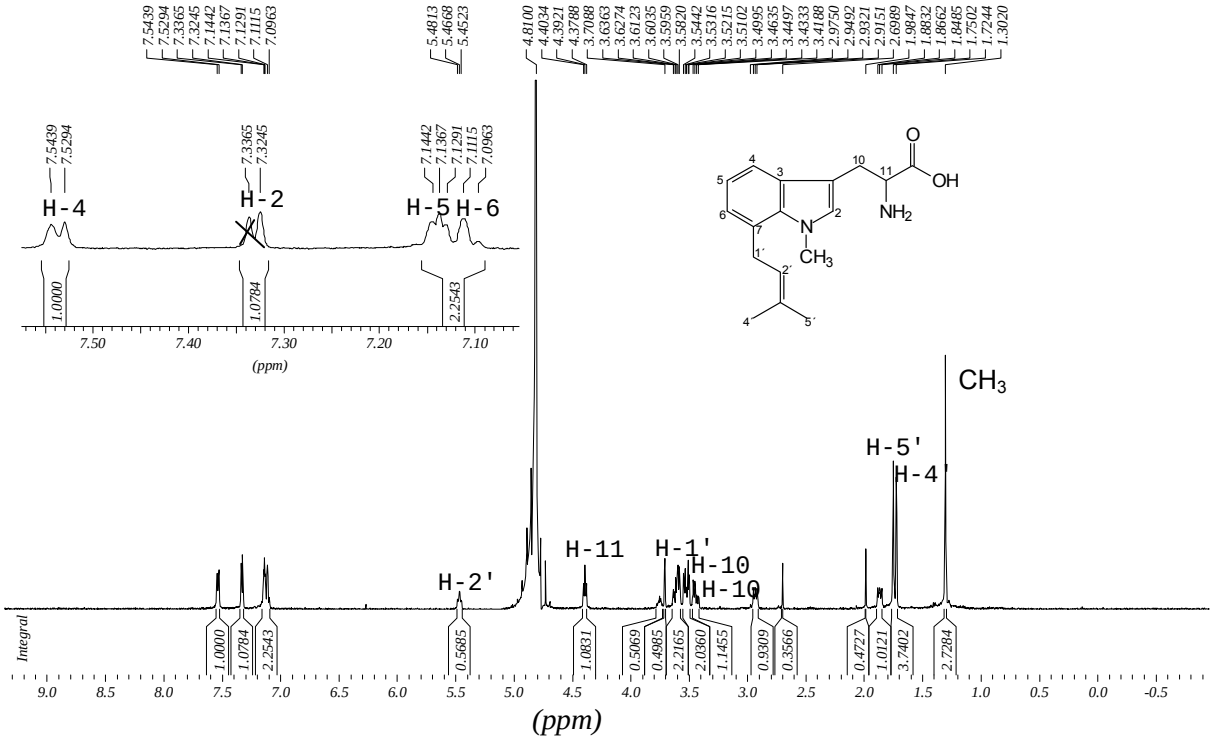


Abb. 4.6: ^{1}H-NMR von 1-Methyl-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

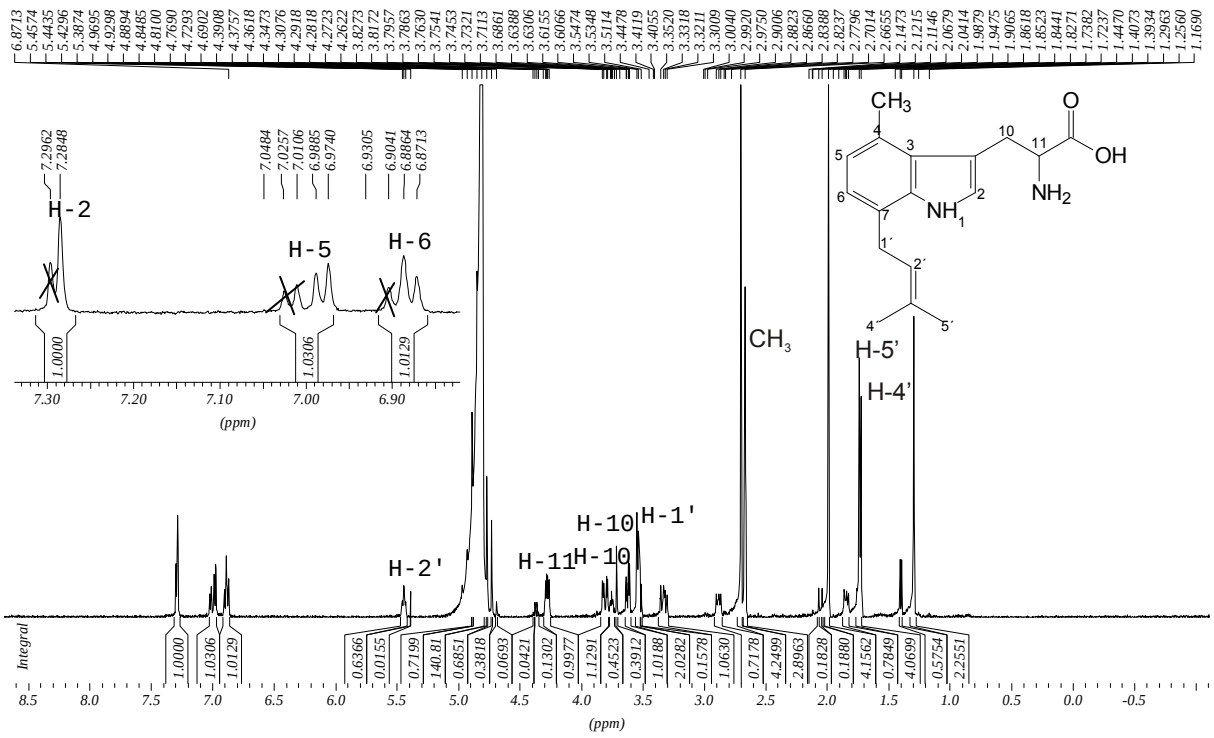


Abb. 4.7: ^{1}H-NMR von 4-Methyl-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

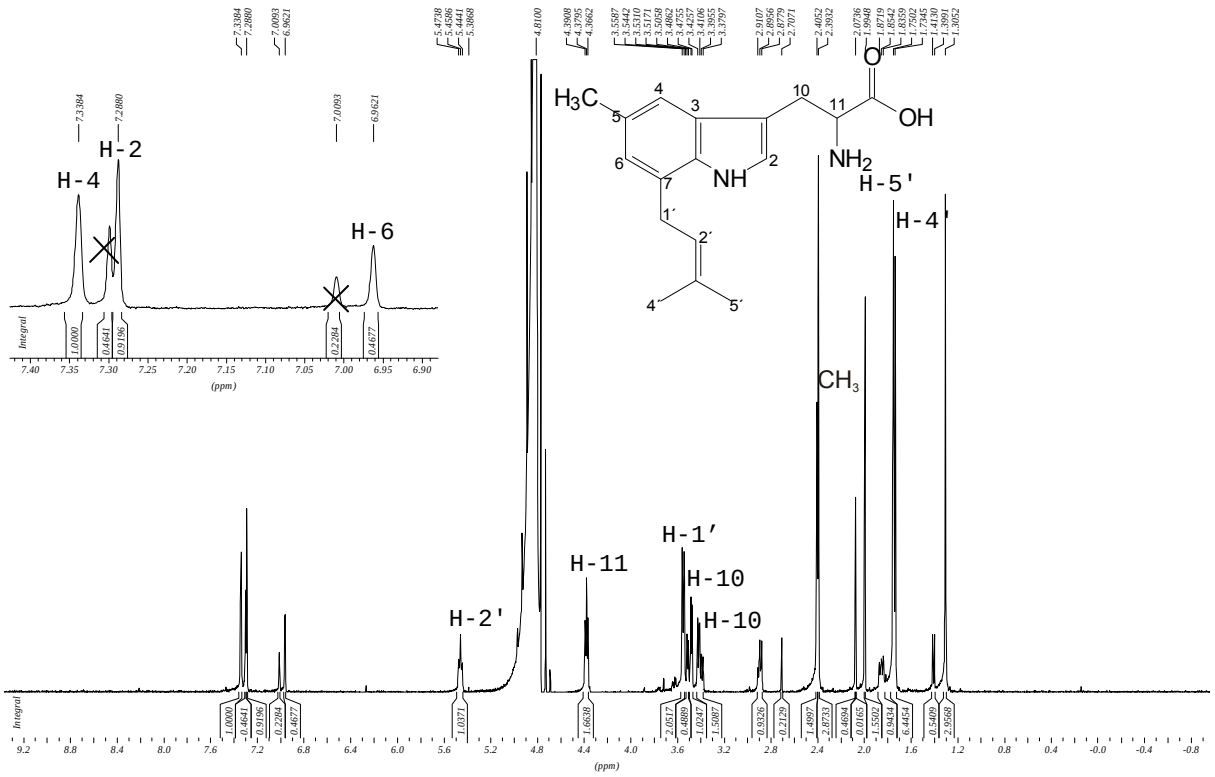


Abb. 4.8: ^{1}H-NMR von 5-Methyl-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

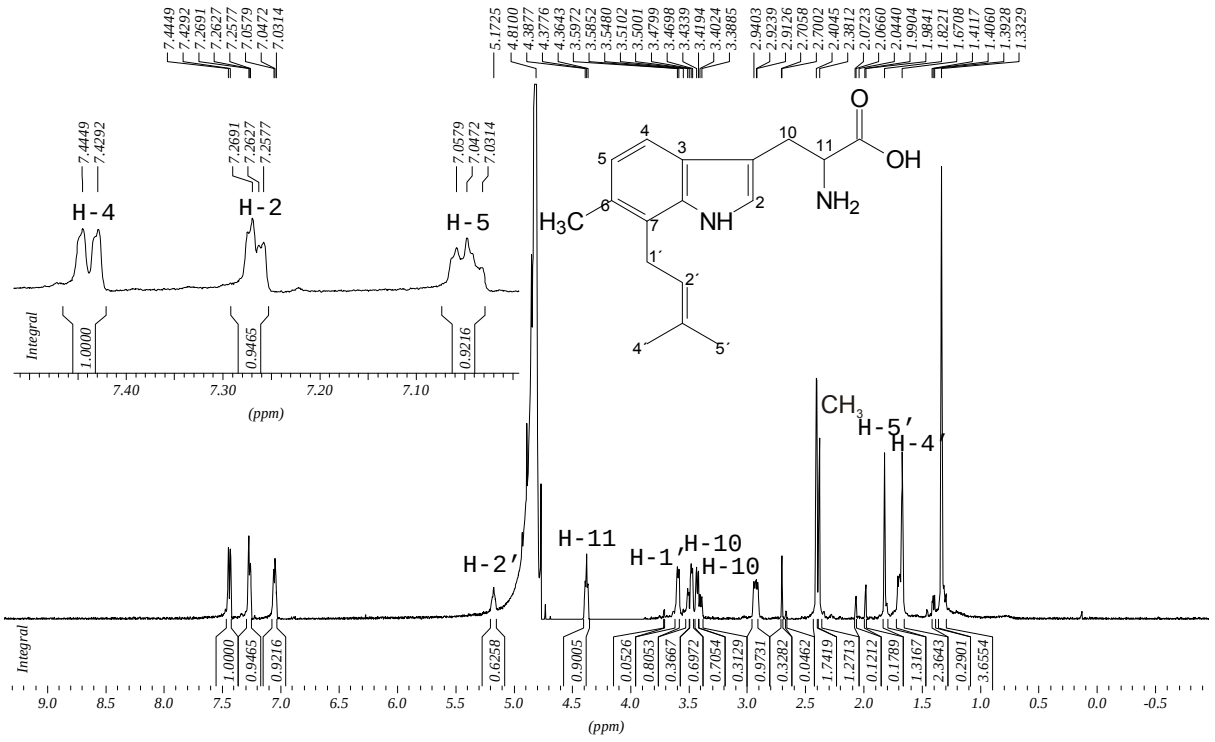


Abb. 4.9: ^{1}H-NMR von 6-Methyl-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

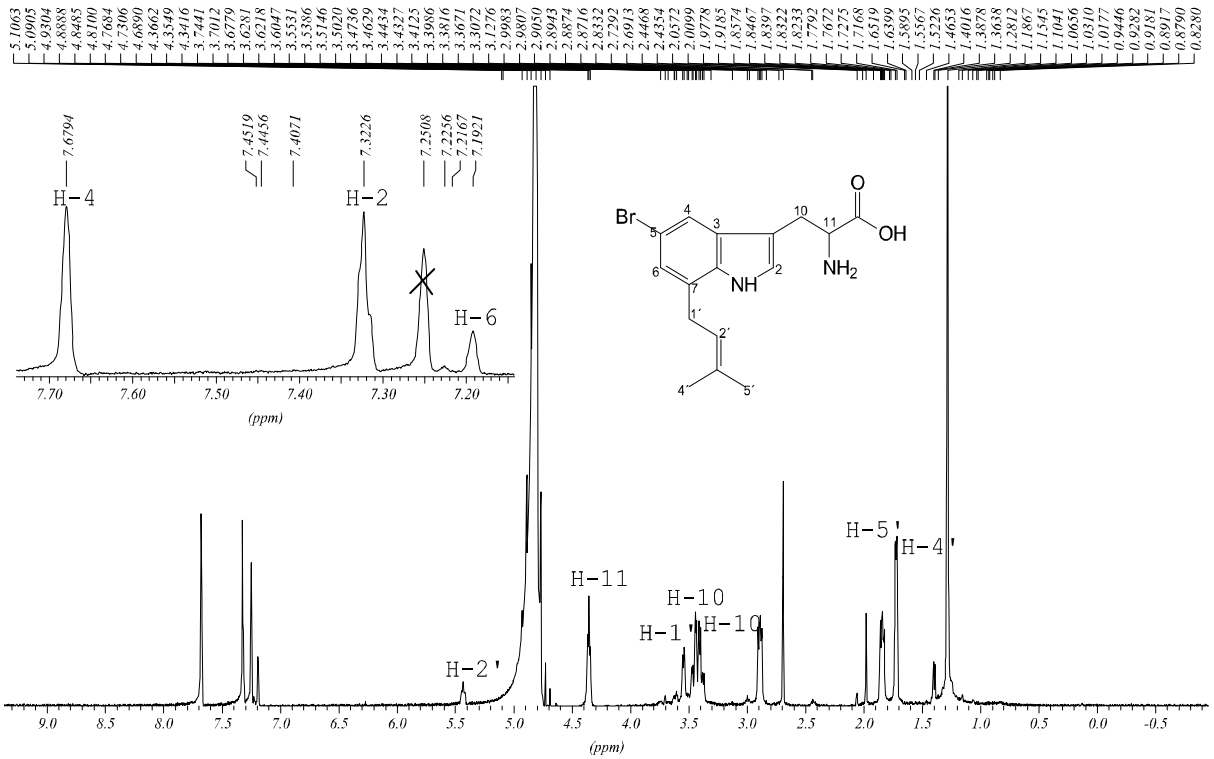


Abb. 4.10: ^{1}H-NMR von 5-Bromo-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

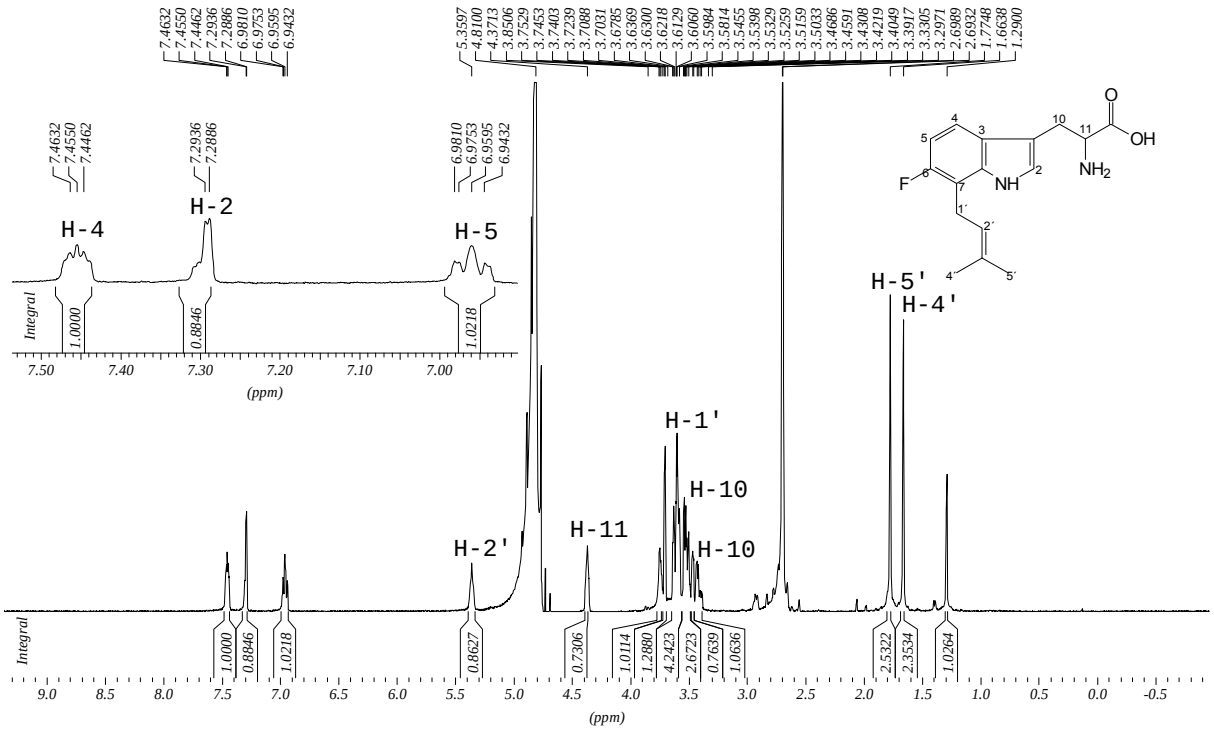


Abb. 4.11: ^{1}H-NMR von 6-Fluoro-7-DMAT, aufgenommen in 0,1 M DCl bei 500 MHz

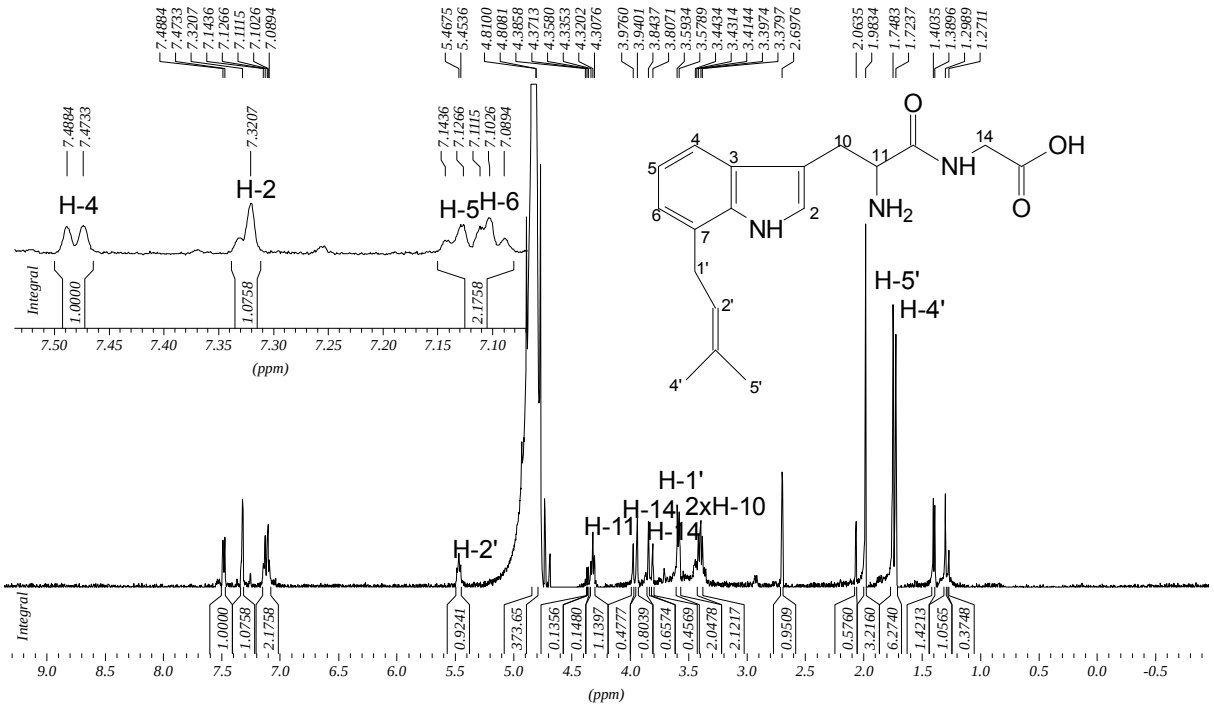


Abb. 4.12: ^{1}H-NMR von 7-DMAT-Gly, aufgenommen in DMSO-d_6 bei 500 MHz

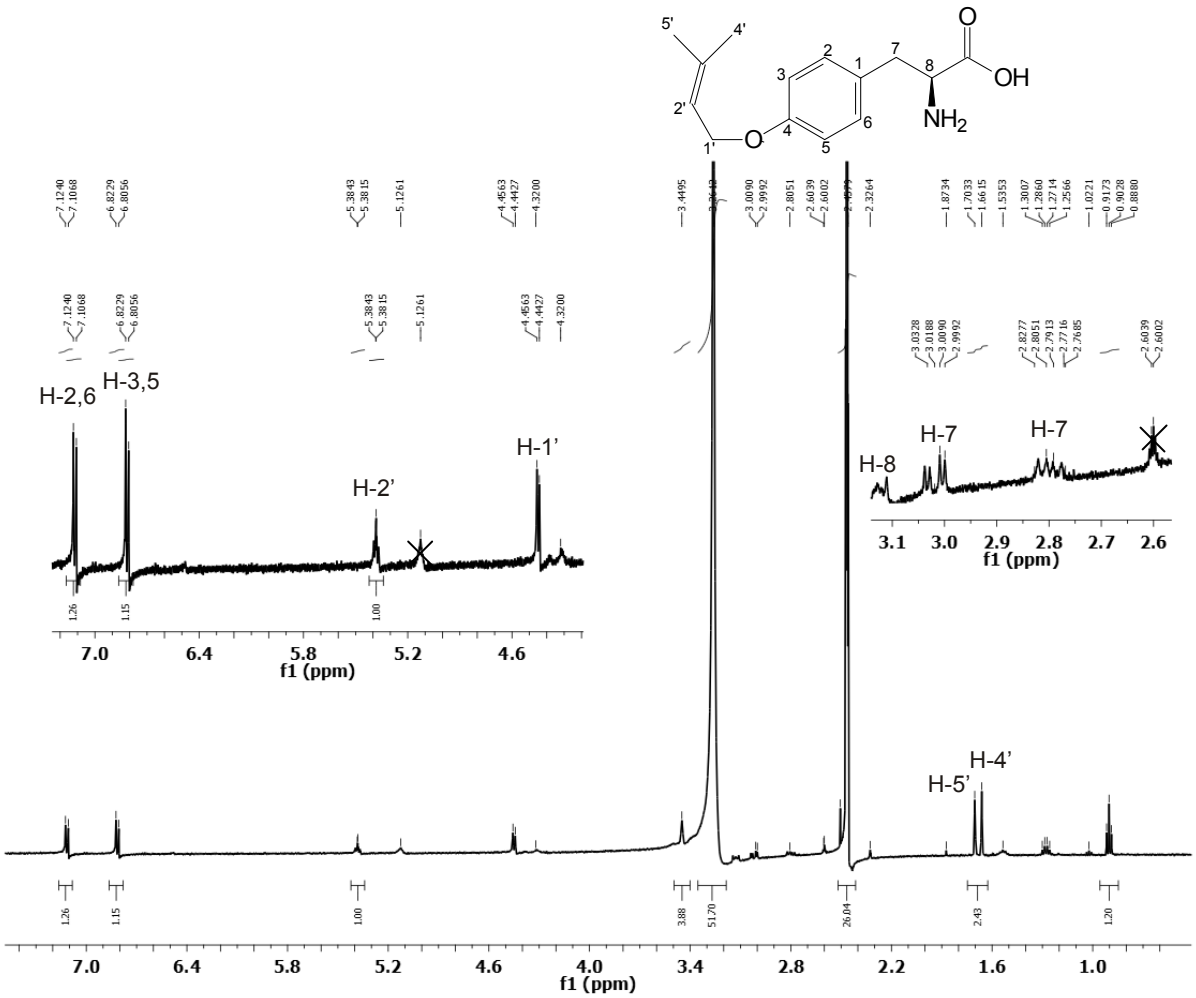


Abb.: 4.13: ^{1}H-NMR von 4-O-Dimethylallyl-L-Tyrosin, aufgenommen in DMSO-d_6 bei 500 MHz

4.3 ESI-MS von verschiedenen enzymatischen Produkten

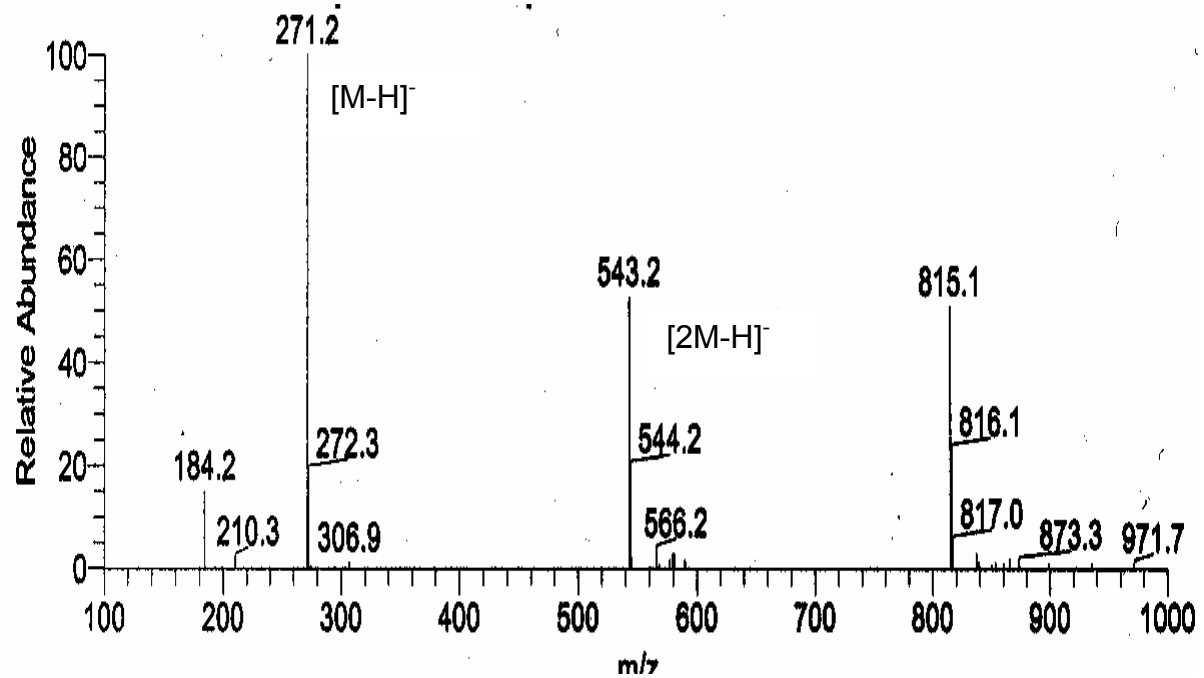


Abb. 4.14: Negatives ESI-MS von 7-DMAT mit dem Massenpeak von 271,2 $[M-1]^-$

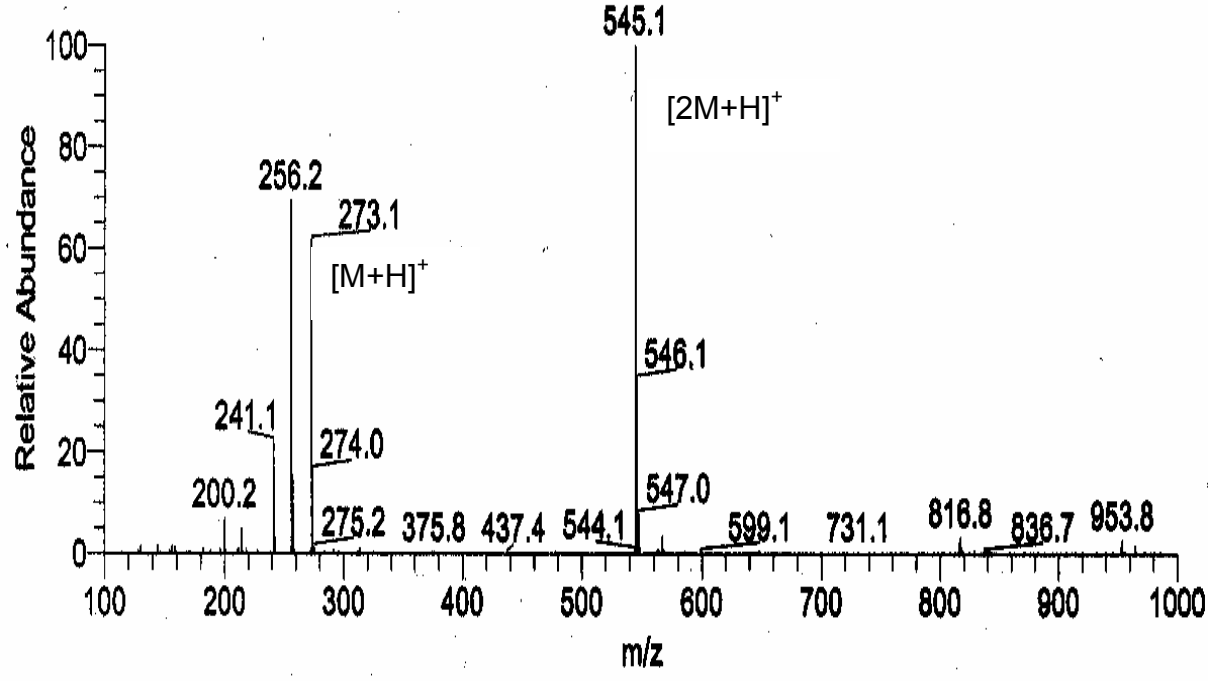


Abb. 4.15: Positives ESI-MS von 7-DMAT mit dem Massenpeak von 273,1 $[M+1]^+$

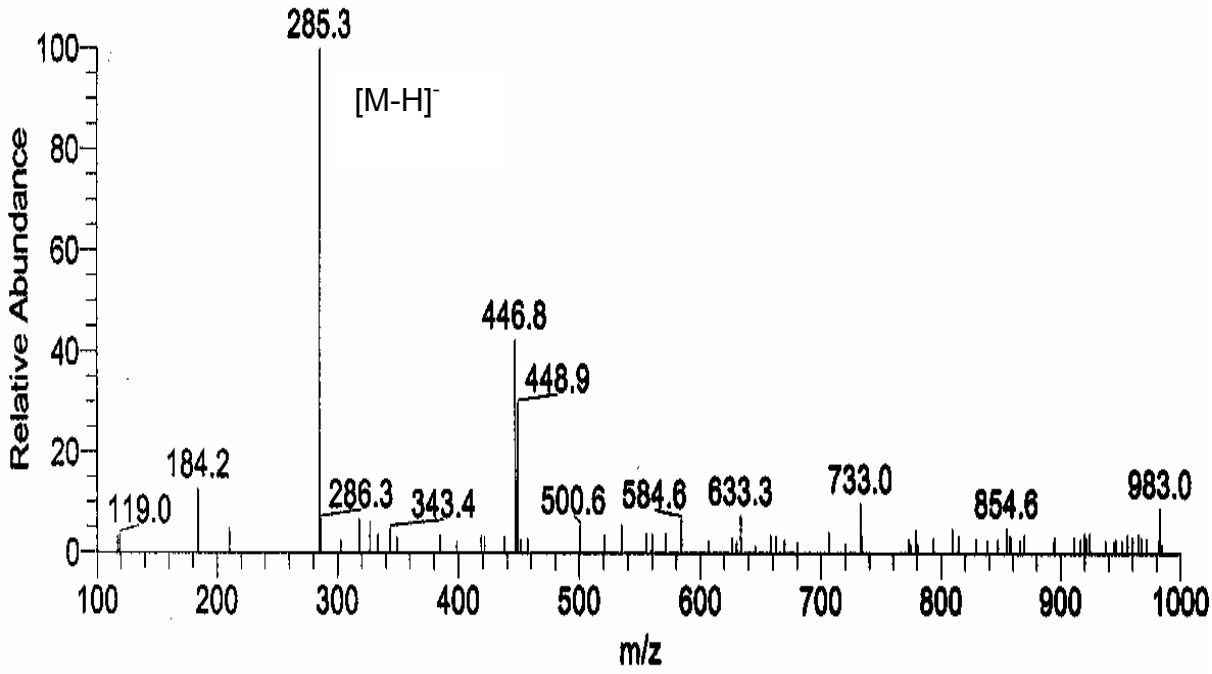


Abb. 4.16: Negatives ESI-MS von 7-DMA-Abrin mit dem Massenpeak von 285,3 $[M-1]^-$

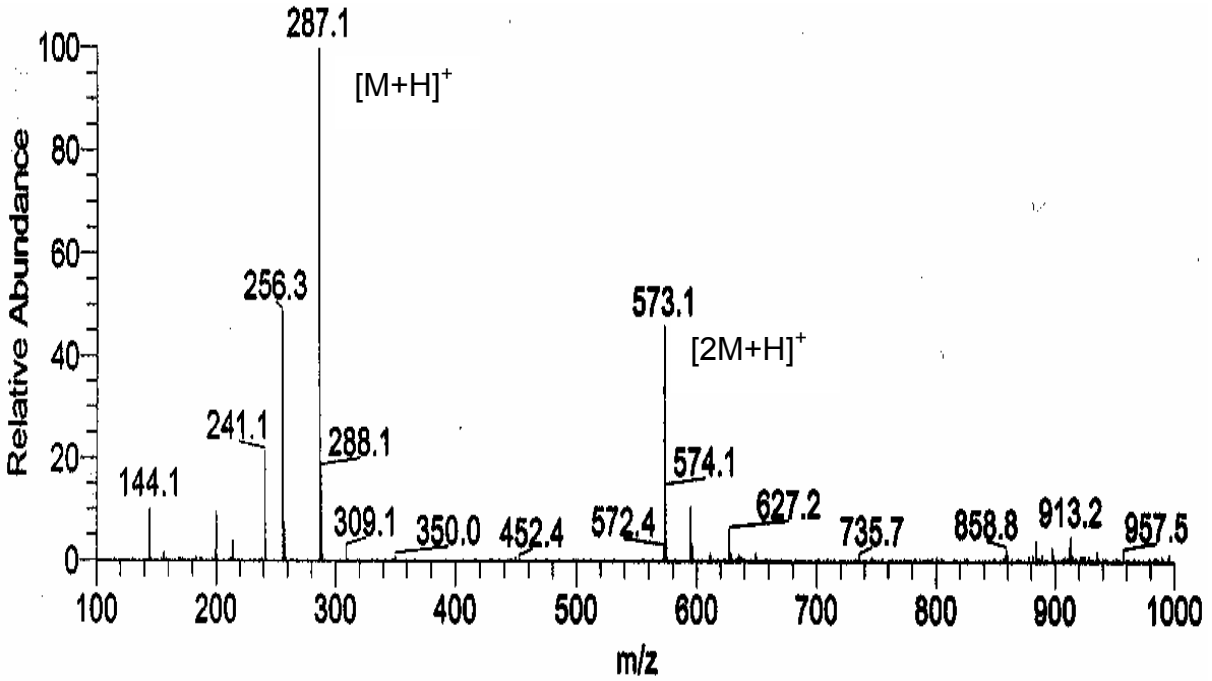


Abb. 4.17: Positives ESI-MS von 7-DMA-Abrin mit dem Massenpeak von 287,1 $[M+1]^+$

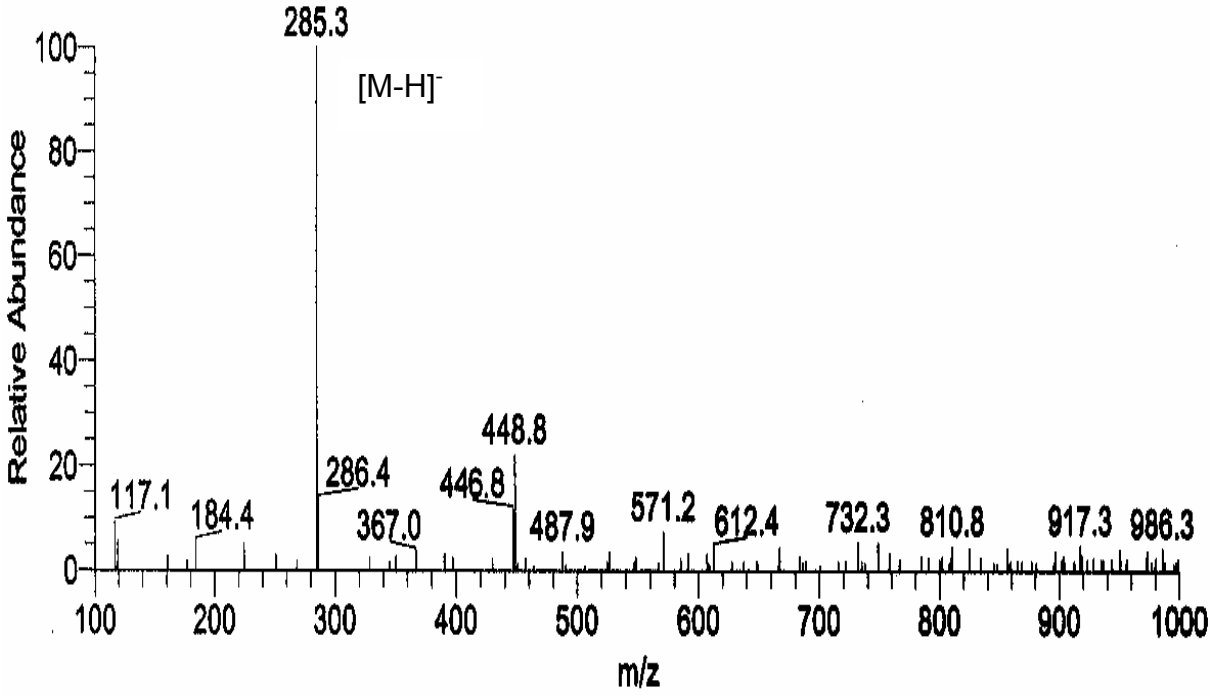


Abb. 4.18: Negatives ESI-MS von 11-Methyl-7-DMAT mit dem Massenpeak von 285,3 $[M-1]^-$

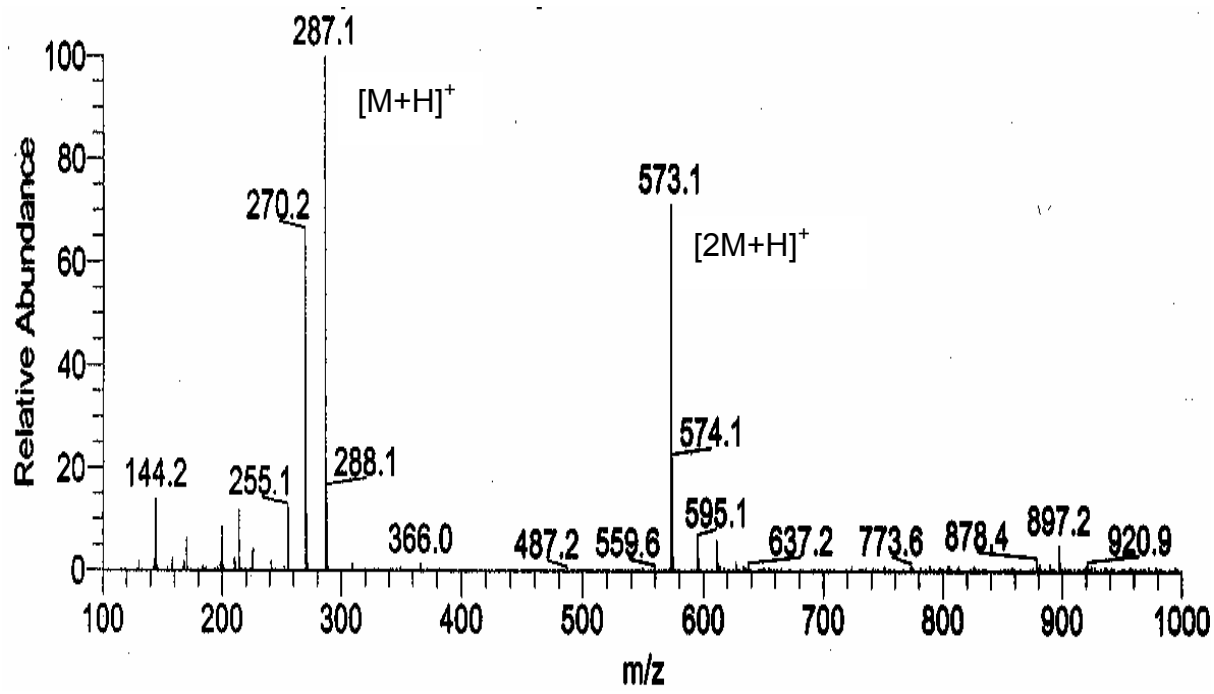


Abb. 4.19: Positives ESI-MS von 11-Methyl-7-DMAT mit dem Massenpeak von 287,1 $[M+1]^+$

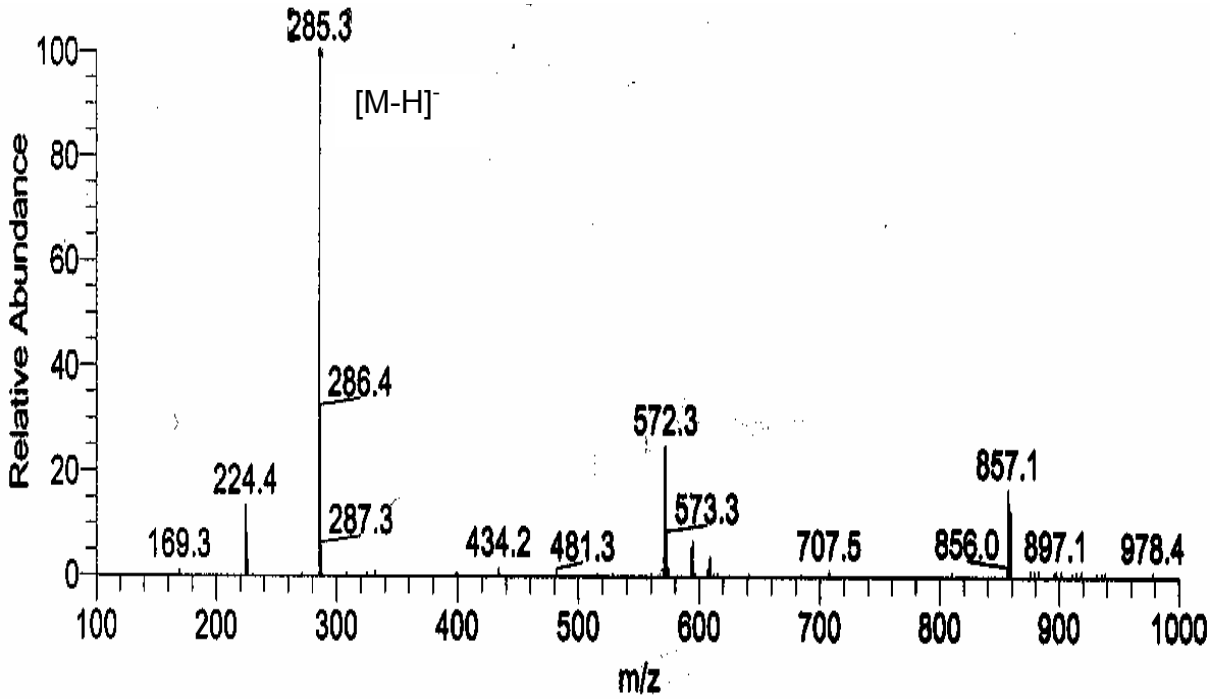


Abb. 4.20: Negatives ESI-MS von 7-DMA-ß-Homotryptophan mit dem Massenpeak von 285,3 $[M-1]^-$

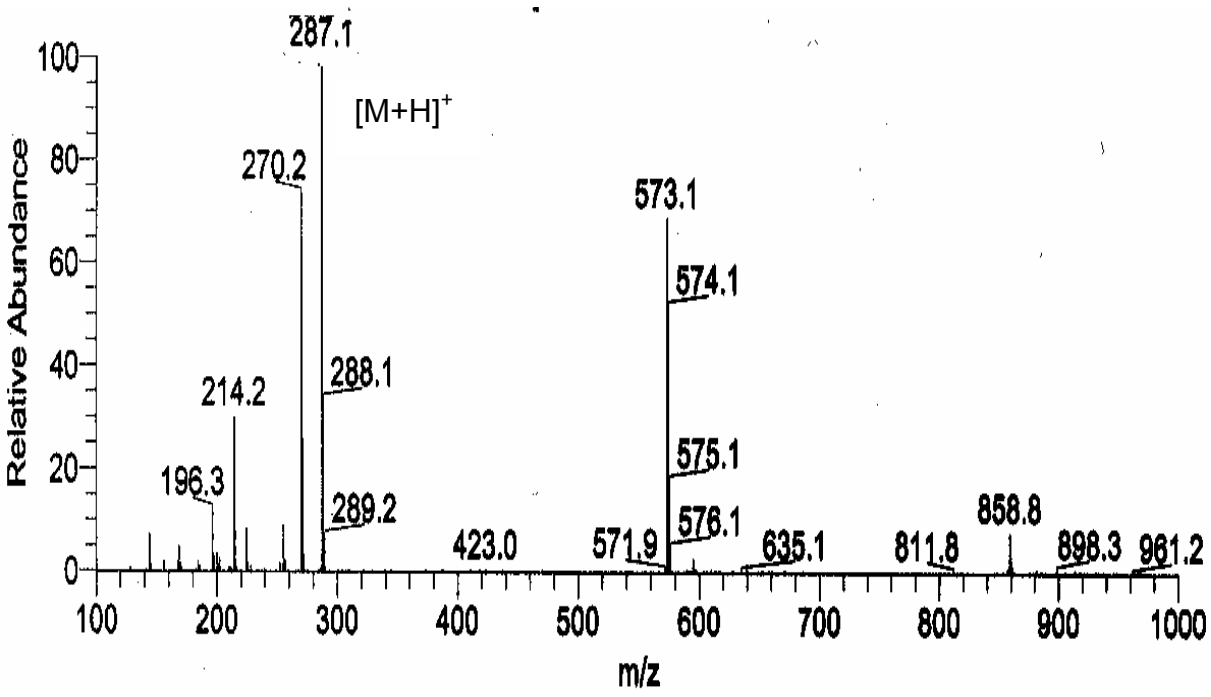


Abb. 4.21: Positives ESI-MS von 7-DMA-ß-Homotryptophan mit dem Massenpeak von 287,1 $[M+1]^+$

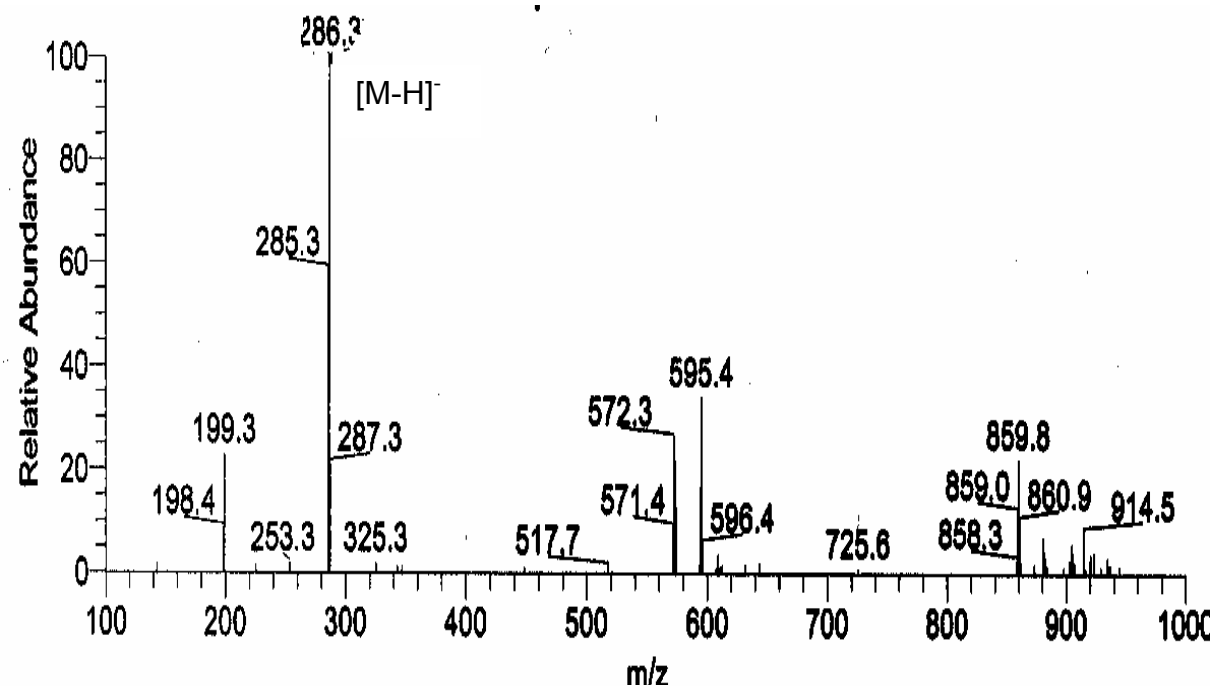


Abb. 4.22: Negatives ESI-MS von 4-Methyl-7-DMAT mit dem Massenpeak von 286,3 $[M-1]^-$

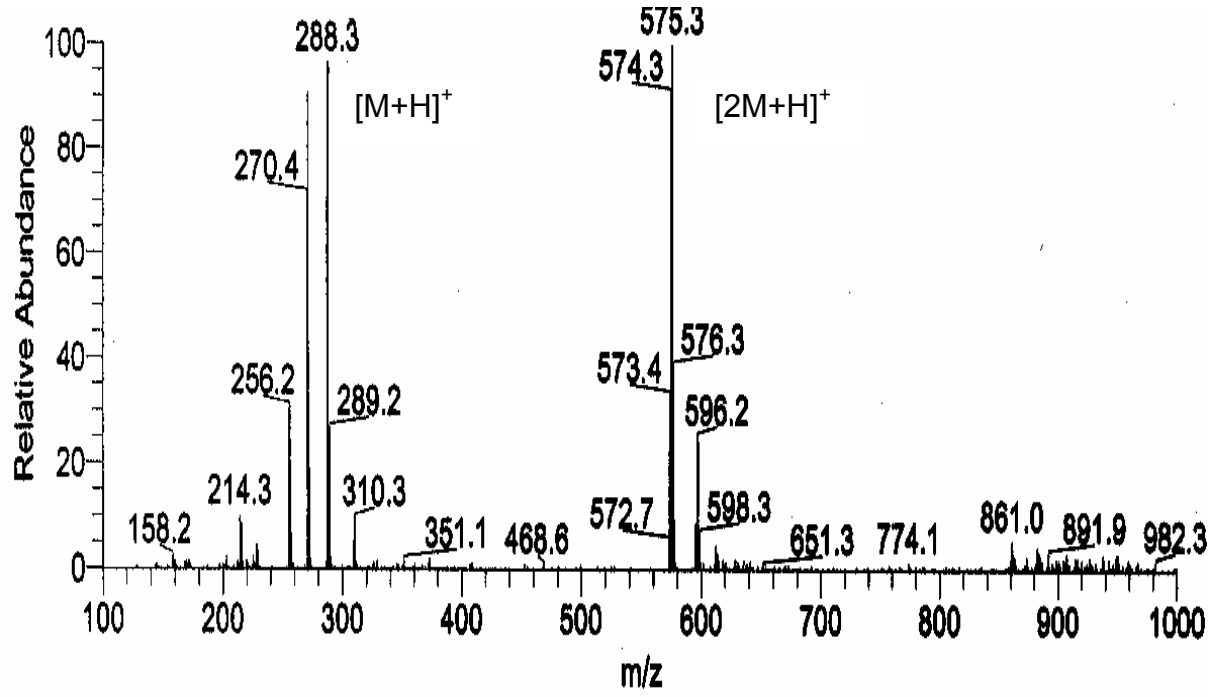


Abb. 4.23: Positives ESI-MS von 4-Methyl-7-DMAT mit dem Massenpeak von 288,3 $[M+1]^+$

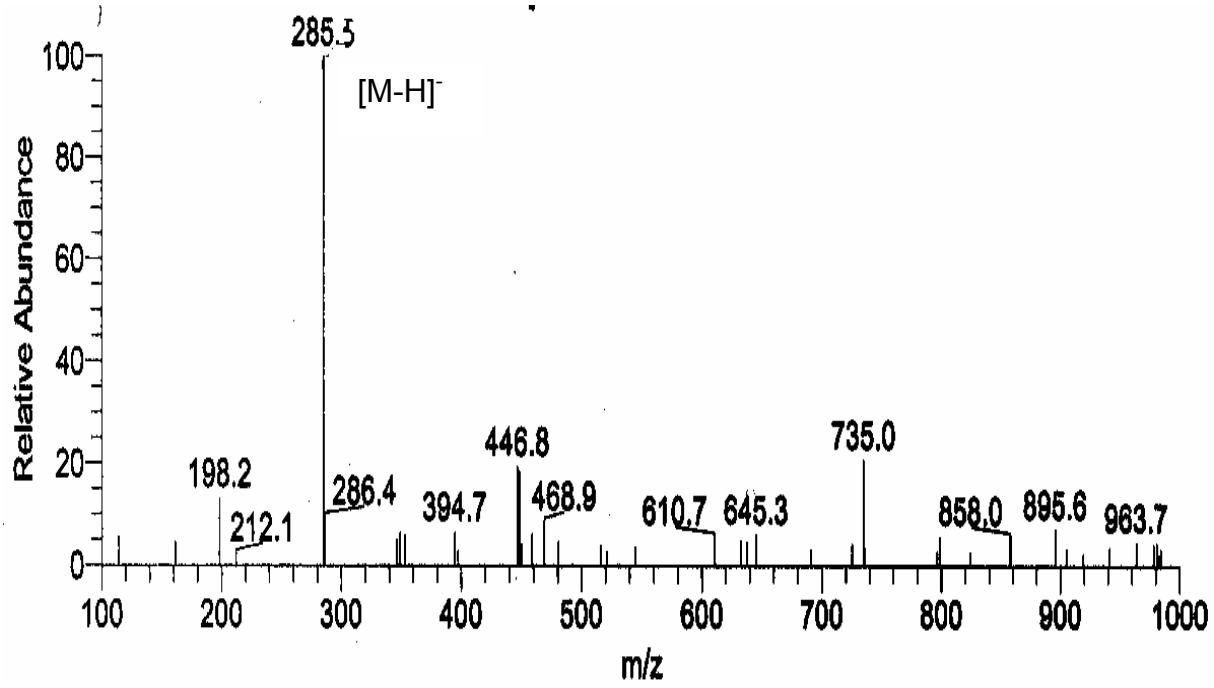


Abb. 4.24: Negatives ESI-MS von 5-Methyl-7-DMAT mit dem Massenpeak von 285,3 $[M-1]^-$

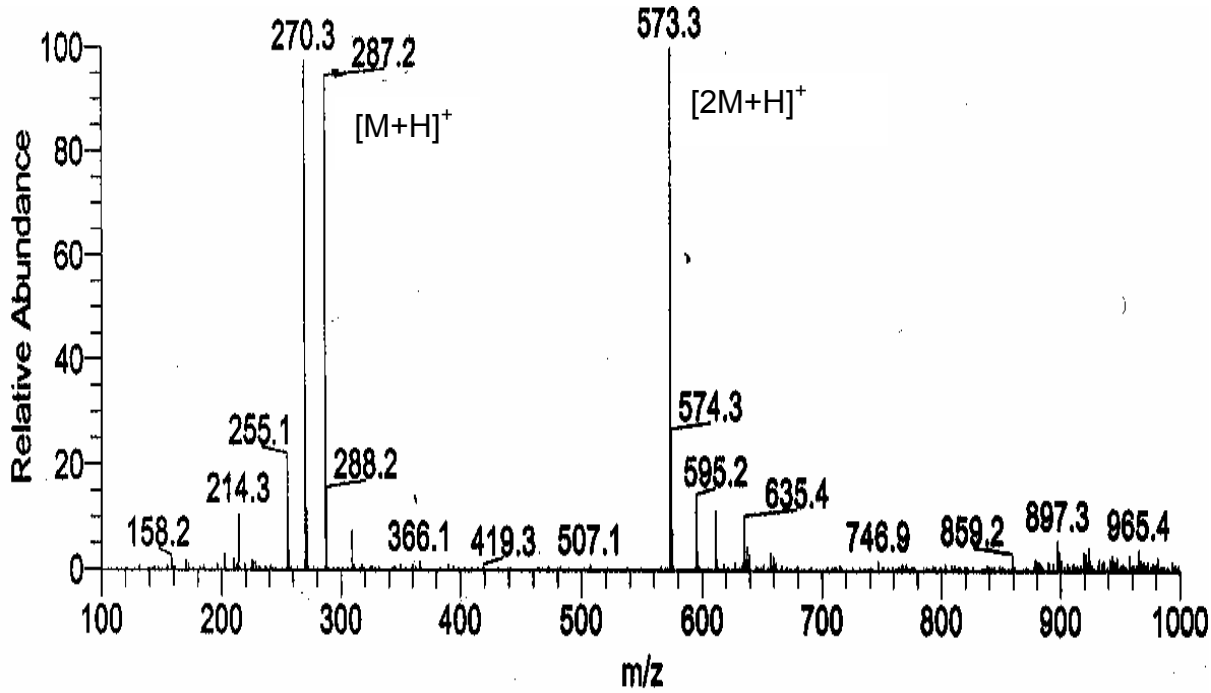


Abb. 4.25: Positives ESI-MS von 5-Methyl-7-DMAT mit dem Massenpeak von 287,3 $[M+1]^+$

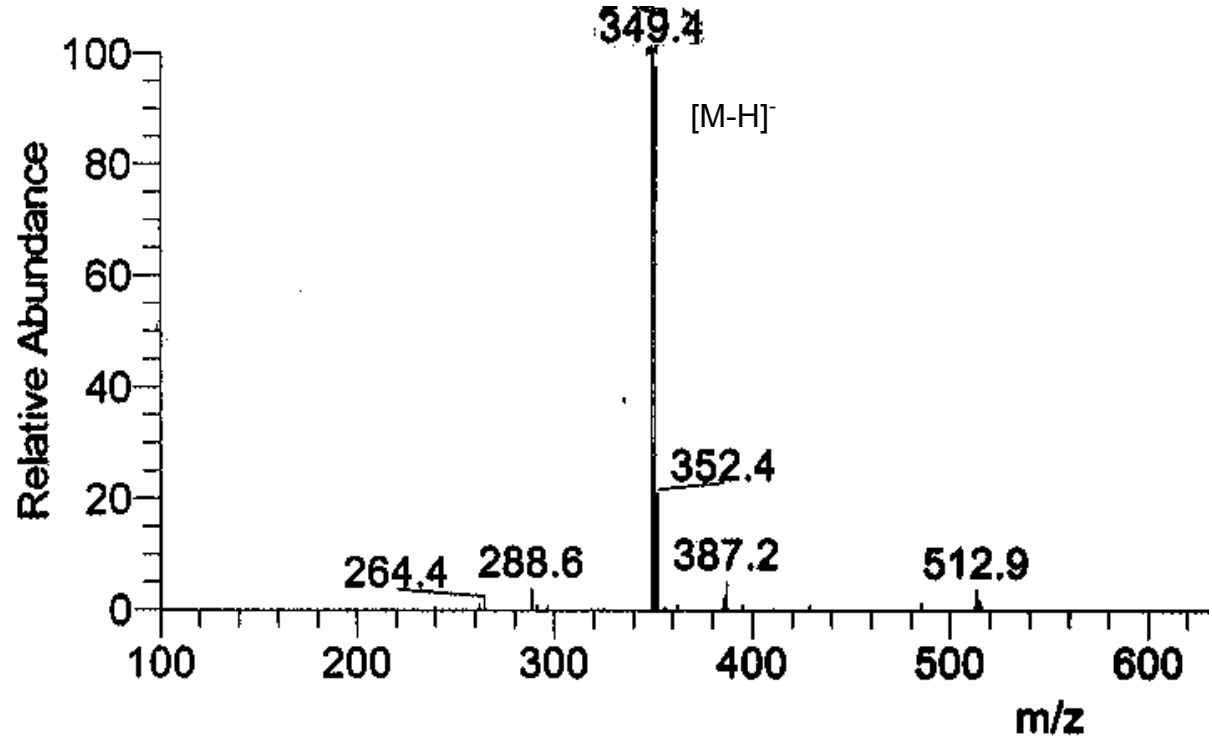


Abb. 4.26: Negatives ESI-MS von 5-Bromo-7-DMAT mit dem Massenpeak von 349,4 $[M-1]^-$

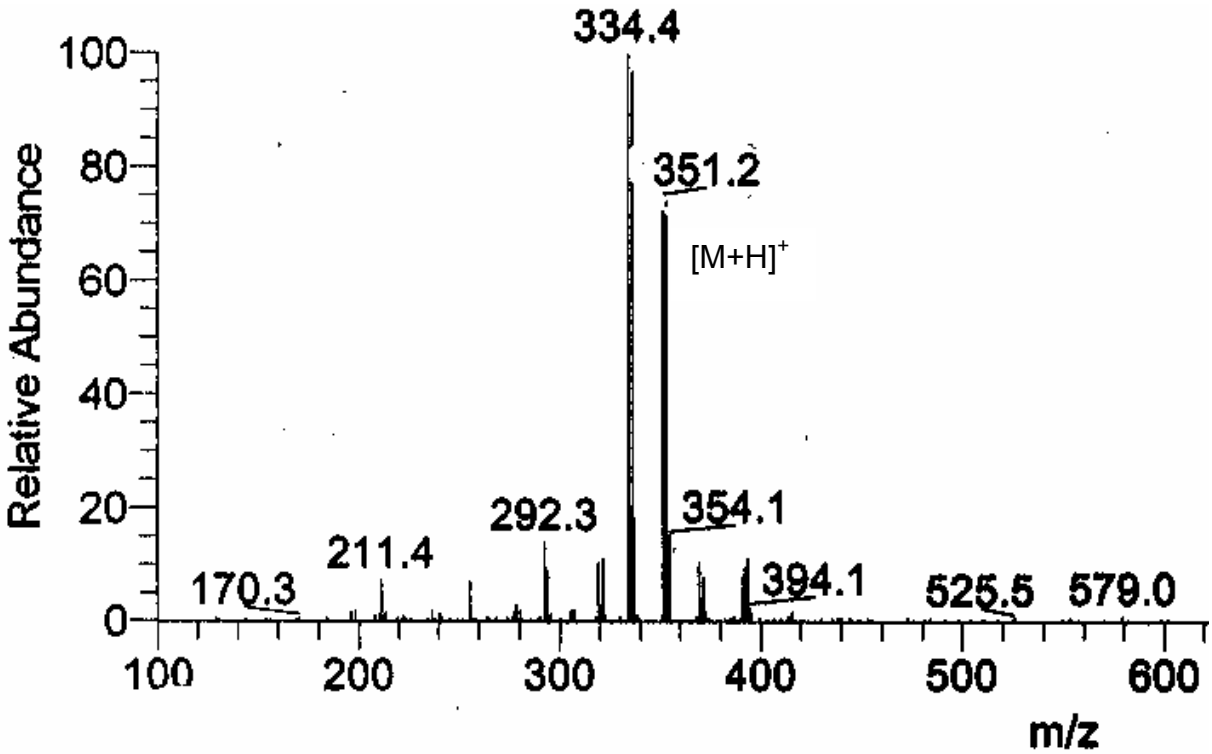


Abb. 4.27: Positives ESI-MS von 5-Bromo-7-DMAT mit dem Massenpeak von 351,2 $[M+1]^+$

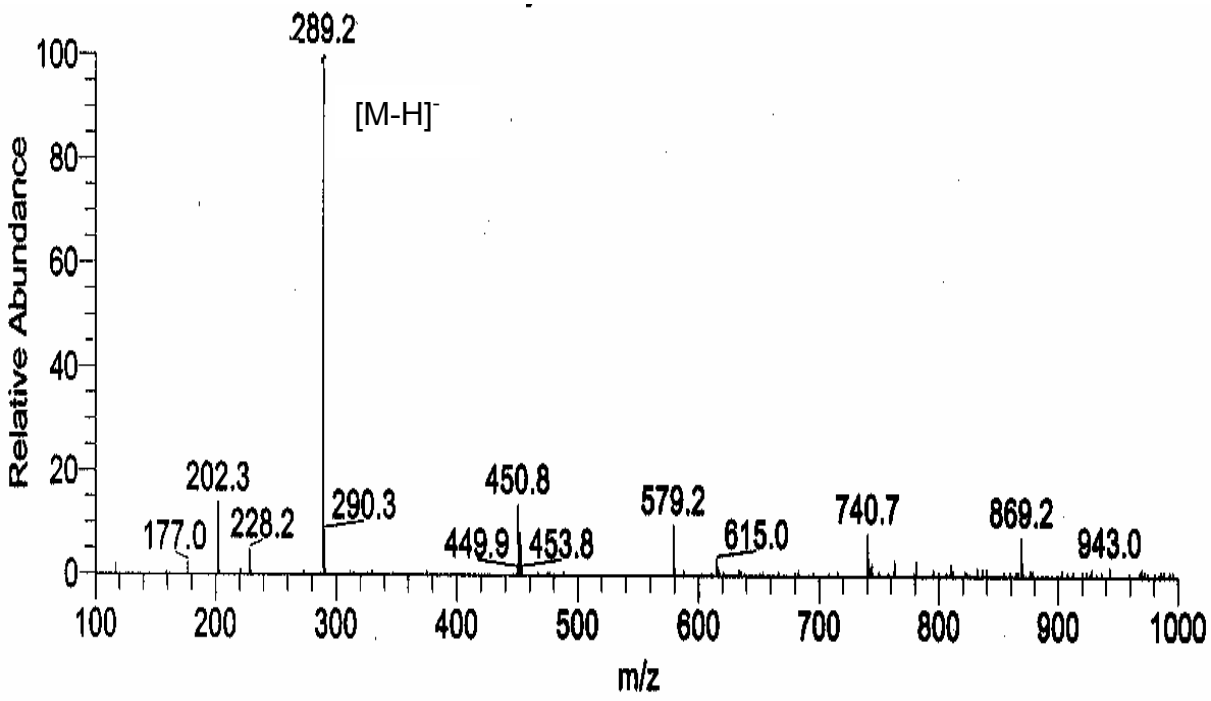


Abb. 4.28: Negatives ESI-MS von 5-Fluoro-7-DMAT mit dem Massenpeak von 289,2 $[M-1]^-$

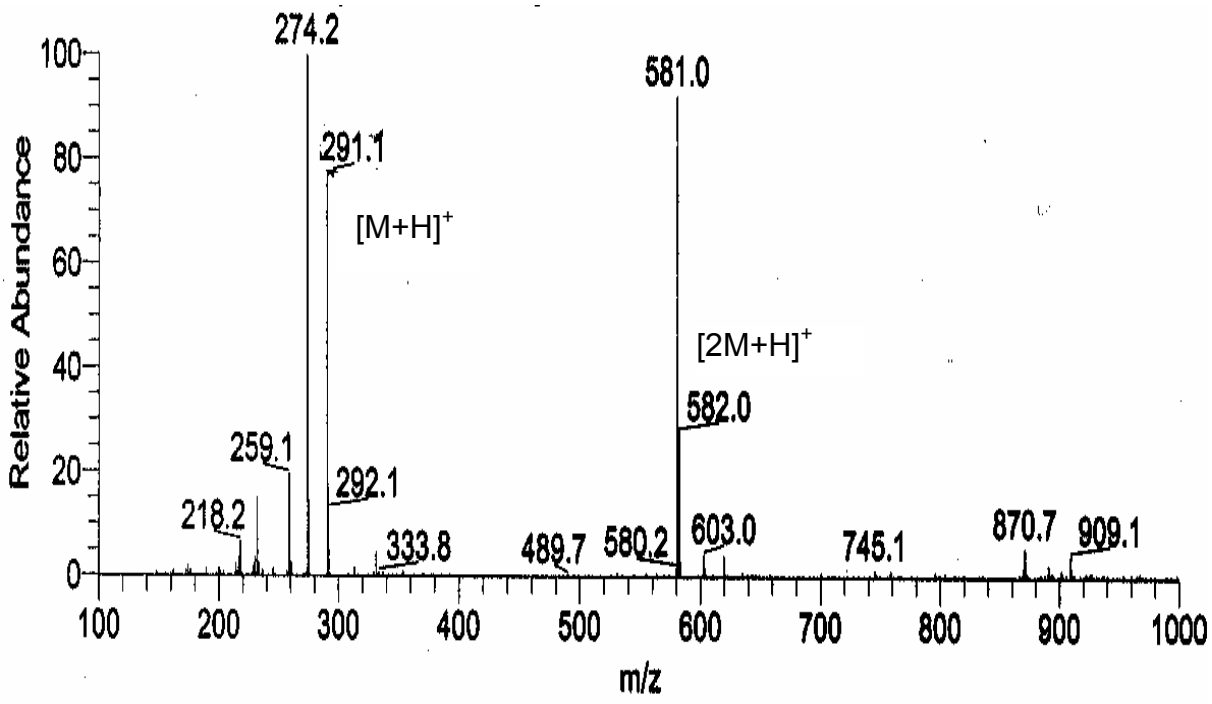


Abb. 4.29: Positives ESI-MS von 5-Fluoro-7-DMAT mit dem Massenpeak von 291,1 $[M+1]^+$

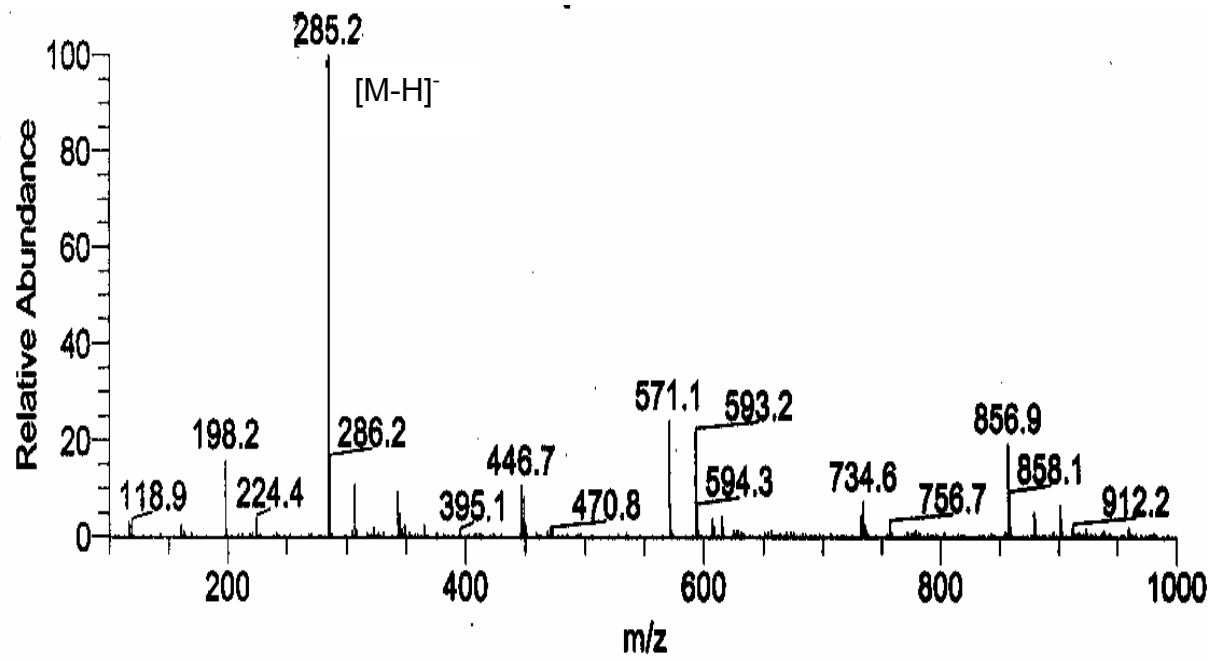


Abb. 4.30: Negatives ESI-MS von 6-Methyl-7-DMAT mit dem Massenpeak von 285,2 $[M-1]^-$

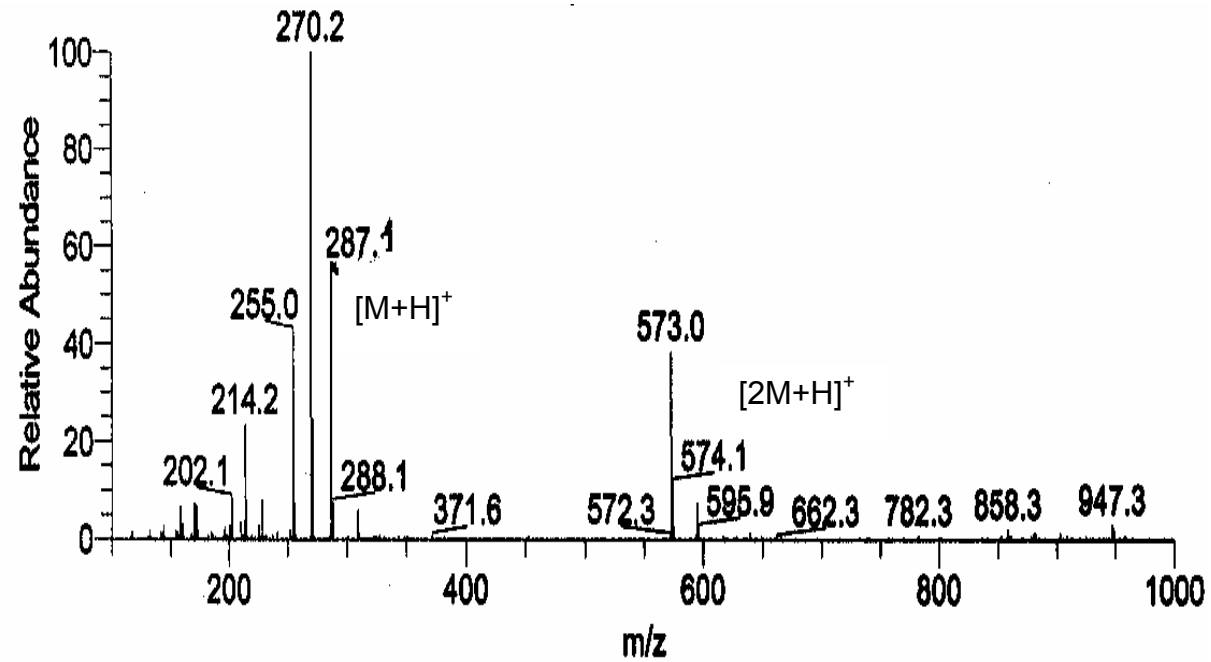


Abb. 4.31: Positives ESI-MS von 6-Methyl-7-DMAT mit dem Massenpeak von 287,1 $[M+1]^+$

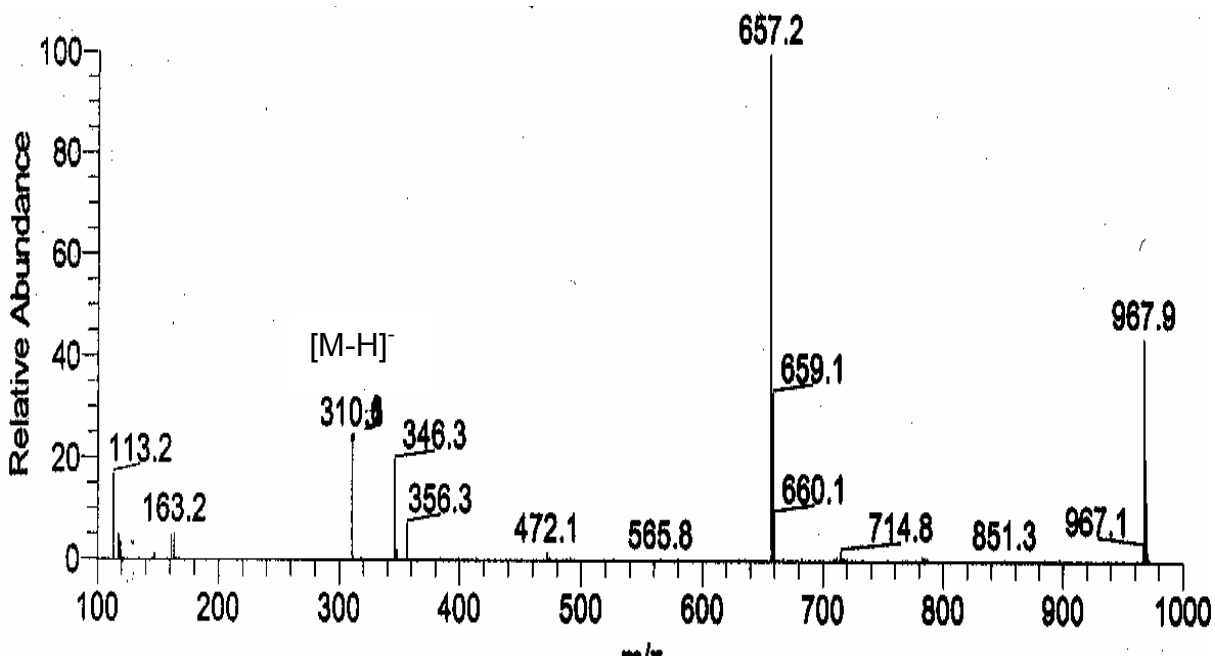


Abb. 4.32: Negatives ESI-MS von cyclo-7-DMAT-Gly mit dem Massenpeak von 310,6 $[M-1]^-$

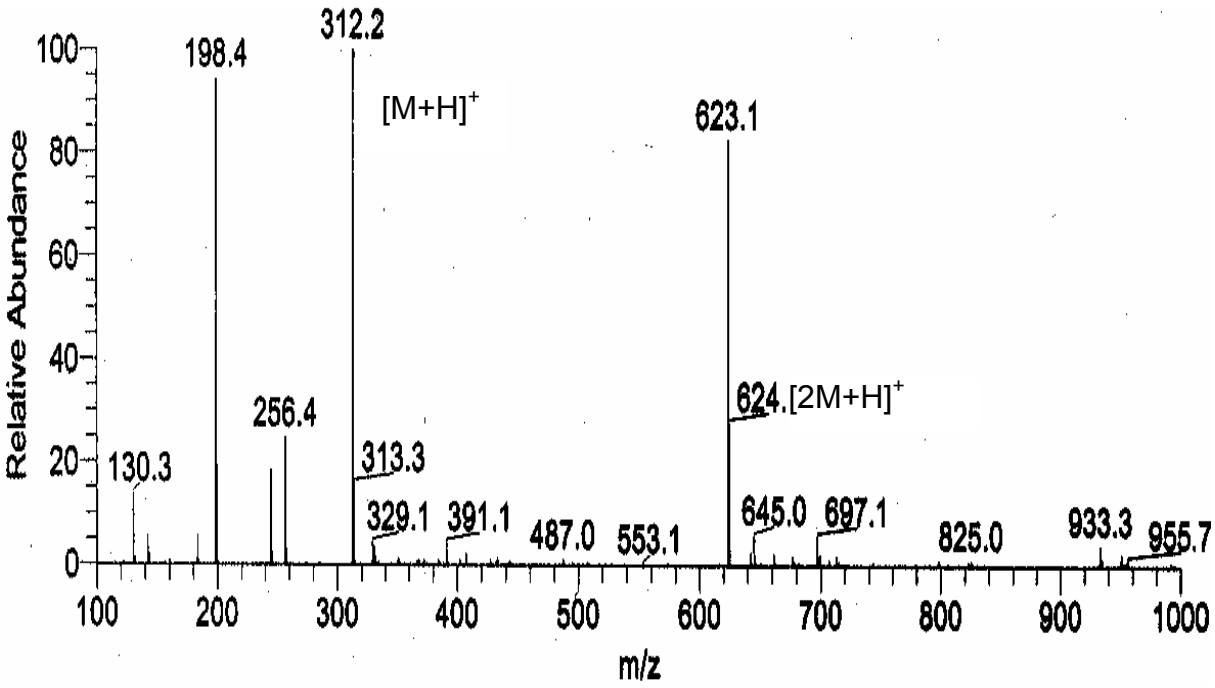


Abb. 4.33: Positives ESI-MS von cyclo-7-DMAT-Gly mit dem Massenpeak von 312,2 $[M+1]^+$

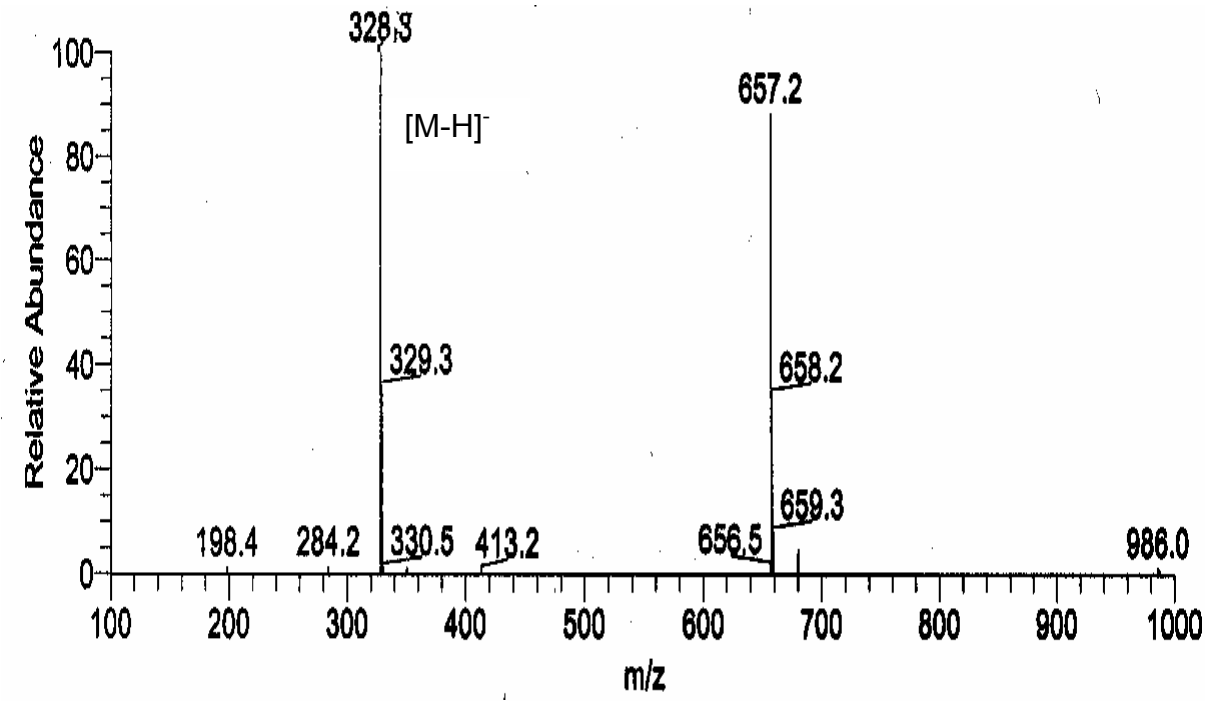


Abb. 4.34: Negatives ESI-MS von 7-DMAT-Gly mit dem Massenpeak von 328,3 $[M-1]^-$

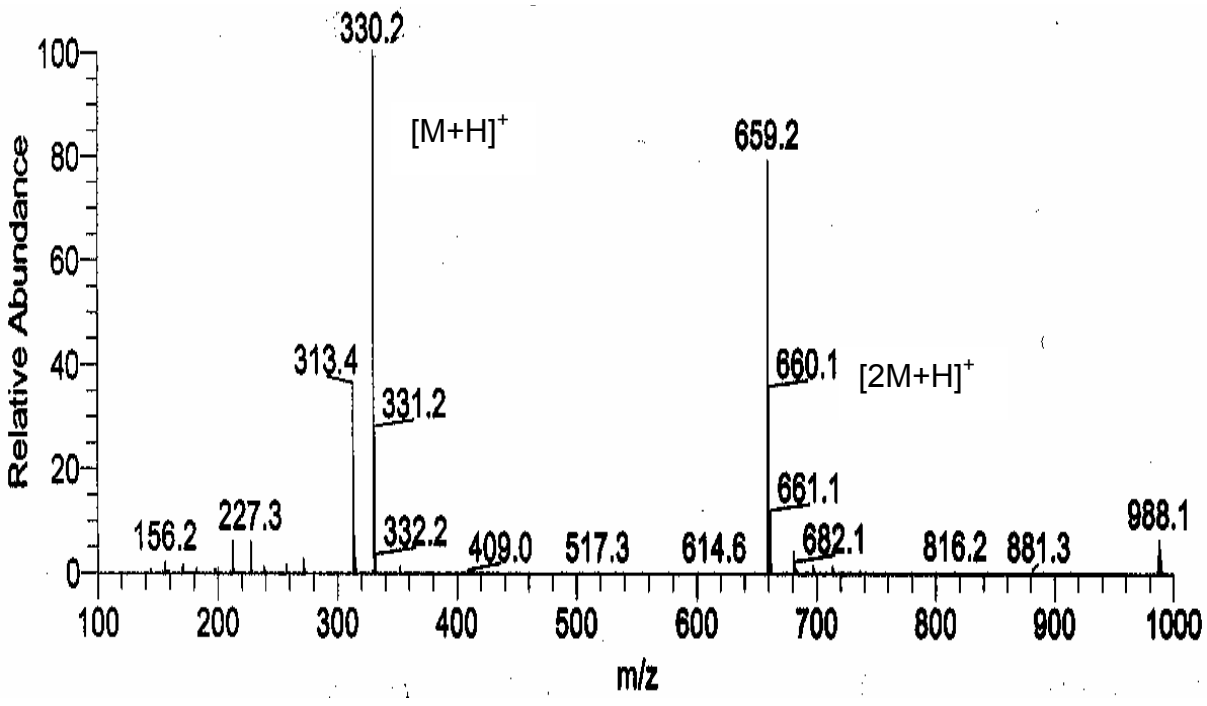


Abb. 4.35: Positives ESI-MS von 7-DMAT-Gly mit dem Massenpeak von 330,2 $[M+1]^+$

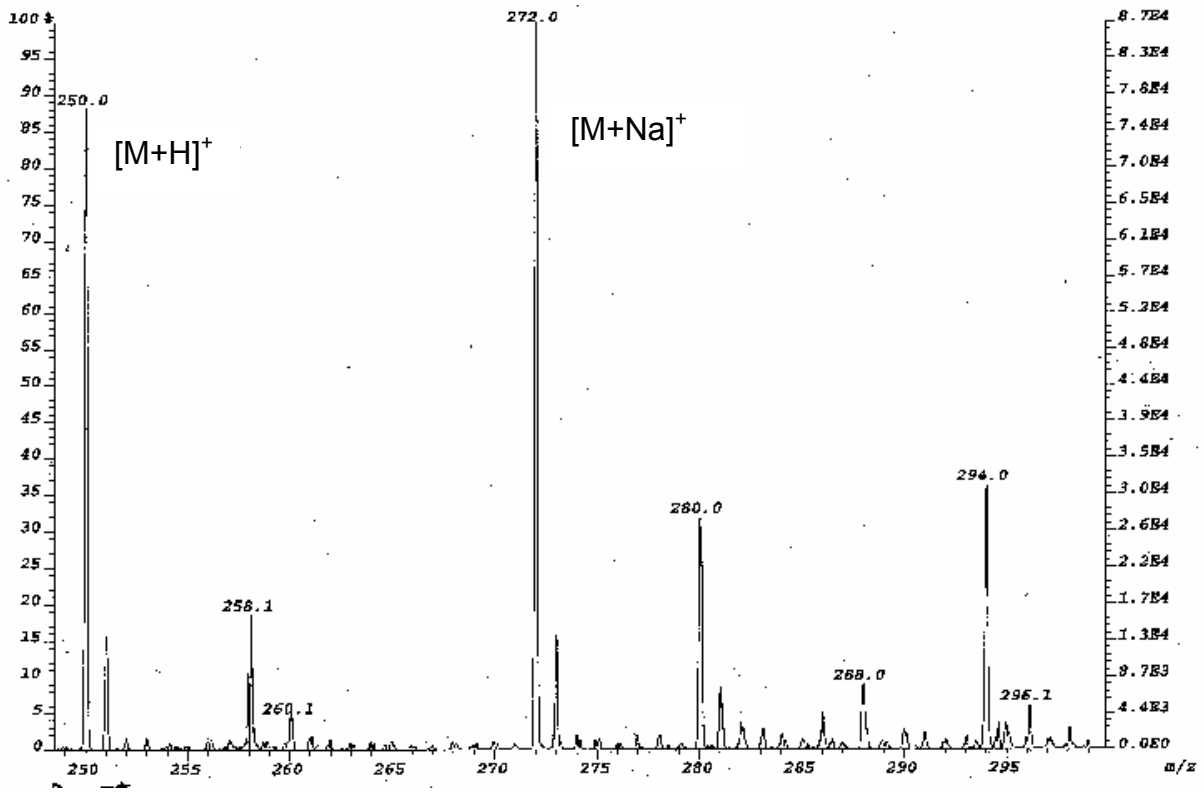


Abb. 4.36: positives ESI-MS von 4-O-Dimethylallyl-L-Tyrosin mit dem Massenpeak von 250 $[M+1]^+$

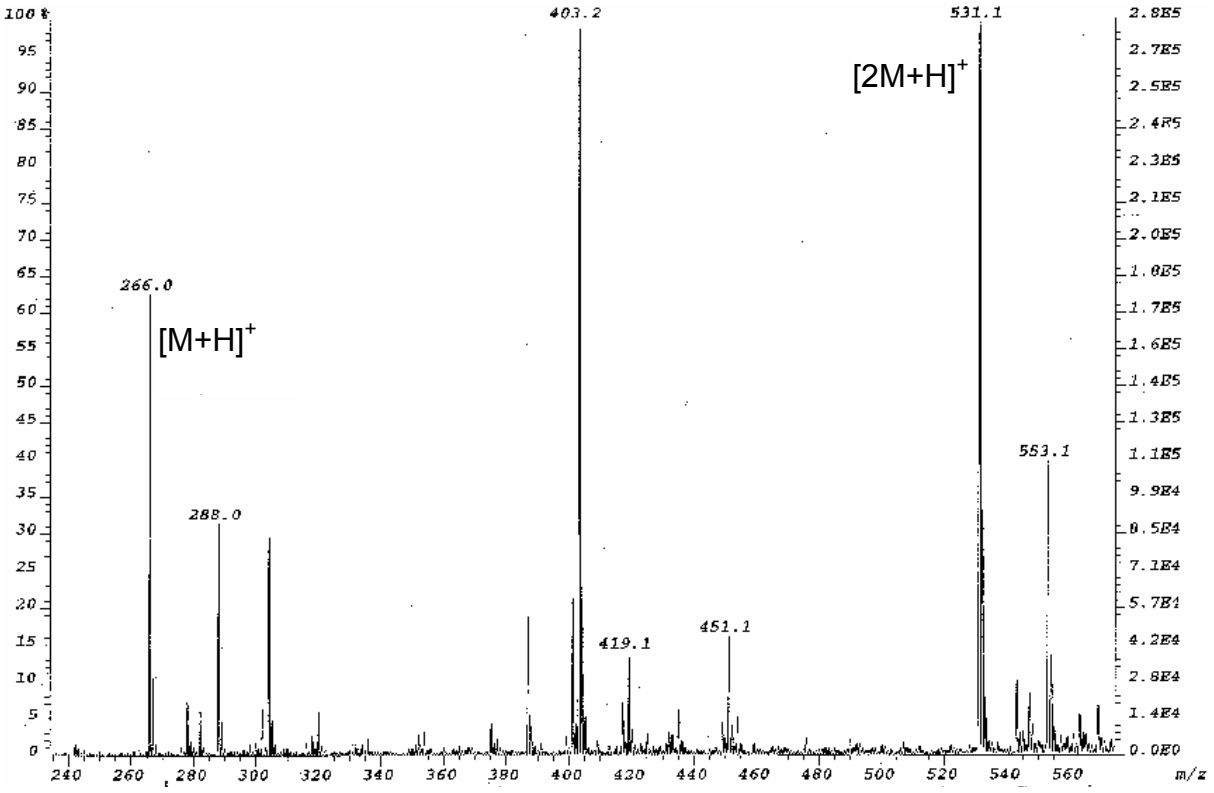


Abb. 4.37: positives ESI-MS von 3-Hydroxy-4-O-Dimethylallylphenylalanin mit dem Massenpeak von 266 $[M+1]^+$

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